UNIVERSITY OF ALBERTA

SPLENIC NEURAL CONTROL OF THE CIRCULATION

BY

KARLI JEAN MONCRIEF ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

FALL 2006

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-22326-0
Our file Notre référence
ISBN: 978-0-494-22326-0

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

The splenorenal reflex modulates cardiovascular regulation and exacerbates renal dysfunction in portal hypertension. Portal hypertension leads to elevated intrasplenic pressure and reduced intrasplenic blood flow, both possible initiating factors for the splenorenal reflex. To distinguish between the two, splenic afferent nerve activity was measured in response to altered intrasplenic hemodynamics in anesthetized rats. To examine central neural involvement in splenorenal reflex-induced cardiovascular effects, *c-fos* expression was quantified in relevant cardiovascular nuclei after splenic vein occlusion in conscious rats. It was concluded:

- Increased intrasplenic pressure (via splenic vein occlusion) stimulates splenic afferent nerve activity through proposed low-pressure splenic baroreceptors;
- Splenorenal reflex activation produces a pattern of central neural activity dependent mainly on direct and indirect input from renal nerves;
- Direct input from splenic afferent nerves is found only in magnocellular neurons of the paraventricular nucleus; therefore, the majority of the spleen's contribution is indirect via peripheral mechanisms.

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my sincerest thanks to my supervisor, Dr. Susan Jacobs, for kindly offering me a home in her research laboratory. The opportunities presented to me both personally and professionally would not have been possible had it not been for her trust and acceptance of me. Her patience, guidance and understanding over the past 3 years have been tremendous, and without, I would not have achieved this much. I will be forever grateful for the foundation of problem solving and critical thinking I have developed under her supervision as a researcher and mentor.

Thank you to the members of my supervisory committee, Dr. Teresa Krukoff and Dr. Peter Smith. Your time and effort, guidance and thoughtful suggestions throughout my graduate career and in reviewing this manuscript have been much appreciated. In addition, thank you to my external examiner, Dr. Samuel Lee, for graciously offering his time for my thesis review and examination.

Many thanks must go to past and present members of the Jacobs' Lab who have contributed in so many ways to this achievement. I am grateful for the opportunity to have learned from and worked with many of you over the last few years. Your friendship and support have not gone unnoticed and will not be forgotten. Thank you for always making my time in the lab a great time.

Great big thanks go to my ridiculously supportive friends. Despite my absence for the better part of a year, they offered nothing but positivity, understanding and encouragement. Thank you for still calling.

Finally, a heartfelt thank you goes to my family for their endless supply of encouraging words, love and support. To my parents, Ron and Jean, for being here for me without actually being here with me; and to my sister, Christie, for watching out for me and seeing me through this one step at a time.

To my family and friends, for all their understanding, love and support.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION TO THE THESIS 1

1.1. HISTORY 2

1.2. STRUCTURE AND FUNCTION 3
  1.2.1. Gross anatomy 3
  1.2.2. Functions of the spleen 4
  1.2.3. Microanatomy 5
  1.2.4. Splenic blood flow 5

1.3. SPLENIC INNERVATION 6
  1.3.1. Sympathetic innervation 6
  1.3.2. Parasympathetic innervation 8
  1.3.3. Afferent innervation 9
  1.3.4. Stimulation of the splenic nerve 9

1.4. PORTAL HYPERTENSION 11
  1.4.1. Cardiovascular system 11
  1.4.2. Renal and endocrine systems 13
  1.4.3. Nervous system 13

1.5. THE SPLENORENAL REFLEX 14

1.6. RATIONALE FOR EXPERIMENTS 15

FIGURES 17
BIBLIOGRAPHY 19

CHAPTER 2. SPLENIC BARORECEPTORS CONTROL SPLENIC AFFERENT NERVE ACTIVITY 29

2.1. INTRODUCTION 30

2.2. METHODS 31
  2.2.1. Animals and housing 31
  2.2.2. Surgery 32
  2.2.3. Splenic vein occlusion 32
  2.2.4. Arterial and venous flow-matched splenic artery occlusions 33
  2.2.5. Extracellular nerve recording 33
  2.2.6. Experimental protocol 34
  2.2.7. Data analysis 34

2.3. RESULTS 35
  2.3.1. Effects of venous and arterial occlusion on blood pressures 35

  2.3.2. Matched reduction in splenic arterial and venous blood flow 36
  2.3.3. Effects of splenic vein and artery occlusions on splenic afferent nerve activity 36

2.4. DISCUSSION 36
  2.4.1. Perspectives 38

2.5. GRANTS 40

FIGURES 41
BIBLIOGRAPHY 48

CHAPTER 3. SPLENORENAL REFLEX MODULATION OF CENTRAL CARDIOVASCULAR REGULATORY PATHWAYS 51

3.1. INTRODUCTION 52

3.2. METHODS 55
  3.2.1. Animals and housing 56
  3.2.2. Drugs 56
  3.3.3. Surgery and chronic instrumentation 57
  3.3.4. Experimental protocol 58
  3.3.5. Tissue processing 59
  3.3.6. Fos immunohistochemistry 60
  3.3.7. Quantification and analysis 60
  3.3.8. Statistical analysis 61

3.3. RESULTS 62
  3.3.1. Hemodynamic measurements 62
  3.3.2. Effect of denervations on numbers of Fos-positive neurons in controls 63
  3.3.3. Effect of splenic vein occlusion on numbers of Fos-positive neurons 64

3.4. DISCUSSION 66

3.5. ACKNOWLEDGEMENTS 78

FIGURES 74
BIBLIOGRAPHY 87

CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS 99

4.1. DISCUSSION 100

4.2. METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS 103

4.3. POSSIBLE FUTURE STUDIES 108

4.4. CONCLUSIONS 109

BIBLIOGRAPHY 110

APPENDIX 114

APPENDIX A. SURGICAL PROCEDURE FOR VESSEL CANNULATIONS 115
APPENDIX B. CONSTRUCTION OF VASCULAR BALLOON OCCLUDER 117
APPENDIX C. ACUTE IMPLANTATION AND USE OF TRANSONIC FLOW PROBES 118
APPENDIX D. PROCEDURE FOR SPLENIC AFFERENT NERVE RECORDING 119
APPENDIX E. CHRONIC IMPLANTATION AND USE OF PA-C10 TRANSMITTER 121
APPENDIX F. INTRACELLULAR PATHWAYS FOR C-FOS EXPRESSION 122

LIST OF TABLES

TABLE 2-1. Baseline values of mean arterial pressure, splenic venous pressure, splenic arterial and venous blood flows and splenic afferent nerve activity from splenic vein-occluded and venous flow-matched and arterial flow-matched splenic artery-occluded groups 42

TABLE 3-1. Baseline blood pressure values from intact, renal-denervated and renal + splenic-denervated groups....................76

LIST OF FIGURES

FIGURE 1-1. Location of the human spleen 17

FIGURE 1-2. Flowchart of the splenorenal reflex pathway 18

FIGURE 2-1. Anatomy of the splenic circulation and configuration of cannulae, flow probes, electrode and balloon occluder 41

FIGURE 2-2. Effects of partial occlusion of the splenic vein or artery on splenic venous pressure 43

FIGURE 2-3. Effects of partial occlusion of the splenic vein or artery on splenic arterial and venous blood flow 44

FIGURE 2-4. Effects of partial occlusion and release of the splenic vein and artery on splenic afferent nerve activity 45

FIGURE 2-5. Raw data trace of splenic afferent nerve activity and splenic venous pressure during splenic vein occlusion 46

FIGURE 2-6. Linear regression analysis of change in splenic afferent nerve activity vs. change in splenic venous pressure 47

FIGURE 3-1. Brain anatomy: nuclei involved in autonomic and neuroendocrine control of the cardiovascular system 74

FIGURE 3-2. Representative photomicrographs showing immunohistochemical staining for Fos in transverse sections of the NTS, VLM, PVN, and SON 75

FIGURE 3-3. Effect of partial splenic vein occlusion on splenic venous pressure in conscious rats 77

FIGURE 3-4. Effect of partial splenic vein occlusion on mean arterial pressure in conscious rats 78

FIGURE 3-5. Effect of denervation and splenic vein occlusion on numbers of Fos-positive neurons in the nucleus of the tractus solitarius 79

FIGURE 3-6. Effect of denervation and splenic vein occlusion on numbers of Fos-positive neurons in the ventrolateral medulla 80

FIGURE 3-7*A*. Effect of denervation and splenic vein occlusion on numbers of Fos-positive neurons in the parvocellular region of the paraventricular nucleus 81

FIGURE 3-7*B*. Effect of denervation and splenic vein occlusion on numbers of Fos-positive neurons in the magnocellular region of the paraventricular nucleus 82

FIGURE 3-8 Effect of denervation and splenic vein occlusion on numbers of Fos-positive neurons in the supraoptic nucleus....................................83

FIGURE 3-9 Representative photomicrographs showing Fos labeling in the hypothalamus of intact splenic vein-occluded animals vs. controls...84

FIGURE 3-10 Flowchart illustrating the expected and proposed central neural effects of the splenorenal reflex 85

FIGURE 3-11 Summary of effects on activation of brainstem and hypothalamic nuclei in response to splenic vein occlusion in intact and denervated animals..86

LIST OF ABBREVIATIONS

ANF	Atrial natriuretic factor
ATII	Angiotensin II
AVP	Arginine vasopressin
CRF	Corticotrophin releasing factor
FLI	Fos-like immunoreactivity
MAP	Mean arterial pressure
MC	Magnocellular
NANC	Non-adrenergic, non-cholinergic
NA	Noradrenaline
NO	Nitric oxide
NPY	Neuropeptide Y
NTS	Nucleus of the tractus solitarius
OVLT	Organum vasculosum of the lamina terminalis
OXY	Oxytocin
PBS	Phosphate-buffered saline
PC	Parvocellular
PVN	Paraventricular nucleus
RD	Renal-denervated
RSD	Renal + splenic-denervated
RSNA	Renal sympathetic nerve activity
SANA	Splenic afferent nerve activity
SAO	Splenic artery occlusion
SFO	Subfornical organ
SON	Supraoptic nucleus
SVO	Splenic vein occlusion
SVP	Splenic venous pressure
VLM	Ventrolateral medulla

CHAPTER 1. INTRODUCTION TO THE THESIS

1.1. HISTORY

The spleen has long been misunderstood, surrounded by an extensive history of fables and myths. This is evidenced by the fact that, in addition to its definition as an abdominal organ, *Webster's Third New International Dictionary* describes the spleen as the seat of emotions and passions; the source of laughter, violent mirth and merriment. The spleen's status as an enigmatic organ has arisen out of much investigation and speculation into its structure, function, and ultimately, its importance.

Some of the earliest writings about the spleen date back to Hippocrates (460-377 BC), who provided the first suggestion of structural functionality for the spleen, by proposing that the spongy organ prevented illness through absorption of an unhealthy excess of fluids (65). It was Galen (121-201 AD) who, through his meticulous anatomic investigation of the spleen and other organs, solidified the structure-function relationship. Although his specific postulations about splenic function did not stand over time, he nevertheless created a foundation in physiology by which organs and organ systems could be discussed as relating to the whole organism (65).

Malpighi (1628-1694) was the first to dispel the mystic properties attributed to the spleen, describing its secretory function as glandular (65). The minute structure of the spleen evaded description until 1696, when Ruysch (following Malpighi's doctrine) described the spleen in likeness to the thyroid, adrenals and lymphatics, as a ductless, vascular gland (65). It was van Leeuwenhoeck (1708) who provided the first detailed descriptions of the spleen as seen by microscopy, and who later identified the spleen's role in purifying the blood (26). This has become the established and most well-known definition for splenic function.

It was the 17th century realization that the spleen could be excised without harm that introduced considerable debate and skepticism over the importance of the spleen for survival

(65). The great philosopher, Aristotle (384-322 BC), even viewed the spleen of minor significance, serving only to physically counterbalance the liver (65). It has only been in the last 50 years that the mentality regarding the non-essential status of the spleen has shifted, with researchers and physicians recognizing the integral role of the spleen in immune defenses and recognizing the risks of post-splenectomy sepsis (27,65). So much has the mentality changed, that the trend in surgical management of splenic disease and injury has been increasingly in favor of splenic preservation over splenectomy (26,27,58,65).

The intent of the following discussion is to further elaborate on the physiological significance of the spleen. Long overshadowed by its well known immunological and hematological functions is the role of the spleen in cardiovascular homeostasis. Evidence will be presented which suggests a mechanism by which the spleen plays an integral role in regulating the cardiovascular system under normal physiological conditions, and conversely, in exacerbating pathophysiological states if the balance is disrupted.

1.2. STRUCTURE AND FUNCTION

1.2.1. Gross anatomy

The human spleen is a relatively fragile organ approximating the size and shape of a clenched fist, extending from the lower epigastric to upper lumbar regions of the thorax (16)(Fig. 1-1). Aside from the hilum, the spleen is covered entirely by visceral peritoneum, forming attachments (ie. ligaments) with adjacent organs such as the stomach, diaphragm, kidney and colon, which function in physically stabilizing the spleen (16). The pulpy interior of the spleen is enclosed by a fibrous capsule composed of elastic and smooth muscle fibers (16). The capsule allows expansion under increased venous pressure, such as that seen after a mea[illegible]ng exercise (16,76). Although containing smooth muscle, the human splenic capsule is relatively non-contractile compared to other athletic mammals such as the dog and horse, which have the capacity to expel high hematocrit blood into the circulation (28,76,83). This property of the human spleen limits its blood storage and expulsion ability (83). Inside the capsule is the trabeculated parenchyma, or pulp of the spleen, which consists of stroma and microvasculature supporting a dynamic expanse of circulating blood and hematopoietic cells (16).

1.2.2. Functions of the spleen

The spleen is the largest secondary lymphoid organ in the human body, having a specialized architecture designed for blood filtration (77). The immunological and hematological functions of the spleen have been clearly established (58,75) and which, without minimizing their importance, will not be discussed in detail here. In brief, the filtering role of the spleen involves clearance of defective erythrocytes, microorganisms, and particles from the blood via the actions of splenic macrophages (16). The spleen plays a unique role in the immune response due to its direct connection with the bloodstream, and acts as a "first responder" to bloodborne antigens through activation and distribution of lymphocytes to the body via the lymphatic system (68). The spleen also has a role in hemostasis, implicated in the storage, synthesis and regulation of hemostatic components such as Factor VIII (Antihemophilic Factor) and IX (Christmas Factor), and in controlling platelet kinetics (16).

In addition to these well-established roles, evidence is accumulating to suggest that the spleen contributes to the regulation of cardiovascular homeostasis through: (a) release of a hypotensive natriuretic factor (29,49); (b) role as 'gatekeeper' for iso-oncotic fluid movement from the intravascular compartment into the systemic lymphatic system (22,46,47); and (c) actions as a peripheral organ involved in the regulation of blood pressure (30,48).

1.2.3. Microanatomy

The microanatomy of the splenic parenchyma reflects its specialized role in blood filtration (Fig. 1-1). The parenchyma comprises white pulp, intermediate marginal zone and red pulp (16). Arterial blood enters the spleen at the hilus via the splenic artery (a branch of the celiac artery), which subdivides into superior and inferior branches known as hilar arteries before piercing the capsule of the spleen (16). Hilar arteries divide as they (along with lymphatic vessels) enter the splenic pulp via trabeculae (16). These trabecular arteries sequentially pass through white pulp to be cleared of lymphocytes, through the marginal zones, which store red blood cells, platelets and monocyte-macrophages, and finally into the red pulp, a network of reticular tissue consisting of capillary beds and venous sinuses (16). Much of the spleen's volume (75%) is red pulp (16), and therefore constitutes the primary site of filtration from the spleen into the systemic lymphatic system. Discontinuous/fenestrated endothelium between the terminal arterioles and proximal veins permits free passage of plasma proteins from the intravascular to extravascular compartments, as evidenced by the iso-oncotic nature of lymph draining from the splenic bed (22,46,47). Venous sinuses drain into trabecular veins, which travel to the hilus where blood exits via hilar veins feeding into the splenic vein, and finally into the hepatic portal vein (16).

1.2.4. Splenic blood flow

The spleen is a highly vascularized organ, receiving approximately 1-10% of total cardiac output, which corresponds to a range of blood flow between 40-100 ml min^{-1} per 100 g of tissue, depending on the species studied (28). The spleen is part of the splanchnic circulation, a significant contributor to cardiovascular homeostasis through its capacity to store a large proportion of the body's blood volume, and to mobilize that store in times of need, such as hemorrhage (36,62).

There are two different routes of blood flow through the spleen, and preference is determined by volemic status. The "fast pathway" transits the majority of blood directly from white pulp to venous sinuses, while only a small percentage of blood flows through the red pulp to reach the venous sinuses (37,56). Arteriovenous shunts allow blood to bypass the filtration zone, significantly speeding passage through the spleen (37,56). The "slow pathway" follows a similar route, but a greater proportion of total splenic blood moves through the red pulp, where a significant amount of plasma fluid is extravasated into adjacent lymphatics (37,56).

In the euvolemic state, the majority of arterial blood passes through the fast pathway (37,38,77), but even through this pathway, approximately one-quarter of cell-free fluid flowing into the spleen is removed from the circulating blood (22). Interestingly, in hemorrhage-induced hypovolemia, the majority of blood passes through the fast pathway (37,56), thereby minimizing further intravascular fluid losses. In conditions of excess fluid, or hypervolemia, the slow pathway is taken preferentially, providing a means to increase fluid extravasation and reduce plasma volume (56). Both direct and simulated volume loading (via activation of atrial volume receptors with an intracardiac balloon) has been shown to reflexly increase fluid efflux from the splenic circulation (22).

1.3. SPLENIC INNERVATION

1.3.1. Sympathetic innervation

Although early reviews of splenic innervation were conservative in reporting neural circuitry of the spleen (70), it is now known that innervation of the spleen is comparable to that of the kidney in terms of afferent and efferent innervation (8). Specifically, there is widespread agreement in the literature over the existence of rich efferent innervation by sympathetic adrenergic fibers. These studies include histochemical and electron microscopic techniques studied in the cat (8,18), cow (51), and rat (2,10-12,69). Felten *et al.* carried out extensive studies in the late 1980's to demonstrate the origin and characteristics of sympathetic innervation of the spleen in an attempt to elucidate the functional link between the central nervous and immune systems (2,3,11,21).

With the use of retrograde tracing, Felten *et al.* confirmed the superior-mesenteric-celiac ganglion as the primary source of post-ganglionic cells innervating the spleen; 98% originate in the superior-mesenteric-celiac ganglion and 2% from the paravertebral sympathetic trunk (8). Furthermore, it was reported that these postganglionic sympathetic neurons provide 95% of total splenic noradrenaline (NA) (11). These studies along with more recent reviews (68,78) provide evidence for a neuroimmune junction through which NA fulfills the criteria for neurotransmission in the spleen with cells of the immune system as targets. Accordingly, activation of the sympathetic nervous system has been linked to controlling the innate and adaptive immune responses (78). Interestingly, endogenous interleukin-1 inhibits NA-induced vasoconstriction of the splenic vessels, an effect requiring intact sympathetic innervation (71). This suggests a role through which the splenic nerves may modulate splenic blood flow and immune cell traffic.

Preganglionic nerve fiber input to the spleen has been traced back to levels $T_6 - T_{12}$ of the spinal cord (11). These fibers pass through lower thoracic sympathetic chain ganglia, traveling with the greater splanchnic nerve (82) to the median and anterior parts of the celiac plexus (2,10,69). The splenic plexus is formed by a dense network of fibers around the splenic artery and its branches (70). These postganglionic nerves are principally vasomotor, innervating the musculature of branching splenic vessels (16). Sympathetic vasoconstrictor fibers are directed primarily to the splenic arterioles rather than venules, providing smooth muscle control over the branching resistance vessels (2,10,69). In species with a muscular splenic capsule, direct sympathetic innervation of the capsule mediates contraction and expulsion of high hematocrit blood in times of physiological stress such as hemorrhage (8).

1.3.2. Parasympathetic innervation

There have been conflicting reports of parasympathetic innervation of the spleen. A virtual lack of choline acetyltransferase (ChAT) activity (known to be a useful marker of cholinergic innervation) led some investigators to conclude that, at best, only a limited number of cholinergic nerves distribute to the rat spleen (12). More recently, however, a review of autonomic innervation of immune organs reported acetylcholinesterase in neuronal and non-neuronal compartments of the spleen suggesting parasympathetic input via the vagus nerve (68). This was addressed by the above mentioned authors, who showed that bilateral subdiaphragmatic vagotomy did not suppress acetylcholinesterase staining, while surgical ablation of the superior-mesenteric-celiac ganglion (removing all ganglionic innervation of the spleen) completely abolished it. In addition, cholinergic cell bodies were found in the superior-mesenteric-celiac ganglion, further confirming the sympathetic ganglion as the primary source of acetylcholinesterase-positive nerve fibers traveling to the spleen, rather than the vagus nerve (12). Due to the lack of measurable acetylcholine in the spleen, the authors propose an alternative role for acetylcholinesterase as a putative neuromediator independent of acetylcholine production and degradation.

1.3.3. Afferent innervation

Although the presence of afferent fibers traveling in the splenic nerve was questioned at one time (69), a number of studies have functionally demonstrated afferent innervation of the spleen, using histological (33,34) and electrophysiological techniques (43,66). The small percentage (~5%) of afferent fibers present in the splenic nerve (8), in comparison to the vast majority of sympathetic efferents, is the likely reason for their absence from earlier literature. The majority of splenic afferent fibers project to levels T_{10}-T_{13} of the spinal cord (8) and resemble unmyelinated C fibers (18,43). A small proportion of myelinated fibers are also present (23,66,67,82) and believed to be sensory, with evidence suggesting a peptidergic neurotransmitter (33,60,61). Splenic afferent nerves have been demonstrated in the guinea pig and appear to be codistributed with substance P– and calcitonin gene–related peptide immunoreactive nerves (33). Similarly, another study found neuropeptide Y–, substance P– and vasoactive intestinal peptide–immunoreactive fibers within and near the spleen (61).

1.3.4. Stimulation of the splenic nerve

Electrical stimulation of the splenic nerve activates NA neurons innervating arterioles, veins, trabeculae and capsule of the spleen (8). Although only half of the postganglionic neurons projecting to the cat spleen have vasoconstrictor properties, reflex activation of the sympathetic nervous system after hemorrhage produces a marked decrease in blood flow and size of the spleen in the cat and dog (8). Additional studies have shown evidence for baroreflex control of splenic nerve activation (13,14,23,70). Opioid peptides are known to coexist with NA in large dense-cored vesicles of the bovine splenic nerve, and may modulate NA secretion in response to sympathetic stimulation (51). Localization of vasoconstrictor efferents to splenic arterioles rather than venules (2,8,10,11,69) suggests a mechanism by which increased sympathetic outflow would cause an increase in the pre- to post-capillary tone of the splenic vasculature, thereby facilitating a reduction in intrasplenic microvascular pressure and consequently reduced fluid extravasation to the lymphatic system. In the relaxed state, up to 25% of splenic inflow is lost to the lymphatic system, suggesting tonic fluid efflux from the intravascular space (22). In species with a non-contractile spleen, such as the human or rat, sympathetic stimulation of the arterioles would therefore contribute to restoring plasma volume, even without expulsion of blood from the splenic circulation.

Unloading of cardiac receptors in vagotomized dogs, as occurs during hemorrhage, has been shown to stimulate splenic efferent nerve activity; generalized sympathoexcitation is observed to offset the significant hypotension and decreased venous return to the heart (53). in contrast, *loading* of carotid sinus baroreceptors in vagotomized cats has been shown to increase splenic volume, presumably through reduced splenic efferent activity (63); furthermore, a reduction in sympathetic efferent activity will preferentially relax splenic arterioles, reducing the pre- to post-capillary tone of the splenic vasculature allowing for increased fluid efflux and a reduction in plasma volume. As evidence, stimulation of cardiac vagal afferents is known to inhibit renal sympathetic nerve activity but have no effect on splenic sympathetic outflow (53), suggesting that it is a lack of sympathetic stimulation which increases splenic volume, not a direct inhibition of nerve activity. The splanchnic organs play a crucial role in mobilizing blood stores, thereby increasing venous return and cardiac output to help restore cardiovascular homeostasis. It is therefore intuitive that a reflex pathway exists between the heart and splanchnic vascular bed (73).

Not only do alterations in cardiac hemodynamics influence splenic neural activity, but the converse has also been demonstrated. Electrical stimulation of splenic afferent nerves produces reflex cardiovascular responses in both dogs (43) and cats (18), including a marked increase in ventricular contractility, heart rate and systemic blood pressure. In addition to the cardiovascular responses observed, *selective stimulation of splenic afferent nerves produces reflex excitation of renal efferent nerves* (18,43). It is this neural reflex between the spleen and kidney, termed the splenorenal reflex, which is the focus of the remainder of this dissertation, in terms of the mechanism by which it operates and its proposed role in normal physiological and pathophysiological states. First, a brief overview will be provided of one such pathophysiological state in which the splenorenal reflex has been implicated – portal hypertension.

1.4. PORTAL HYPERTENSION

1.4.1. Cardiovascular system

Portal hypertension is a complex condition characterized by many hemodynamic disturbances, often resulting in severe renal and cardiovascular dysfunction. The condition is defined by an elevated hepatic portal venous pressure above its normal physiological range (ie. >10mmHg) (39,41). The pathological increase in portal venous pressure and therefore increased intrahepatic resistance may result from a prehepatic, intrahepatic and/or posthepatic obstruction in the venous drainage system of the liver (39,41). Variation in the site of increased resistance to portal venous flow contributes to the etiological differences observed in these portal hypertensive syndromes, although there are considerable similarities among them (39,41). Portal hypertensive patients present clinically in a consistent manner, exhibiting a hyperdynamic circulation typified by an increased blood volume and cardiac output, a reduction in total peripheral resistance and mean arterial pressure, and a marked increase in splanchnic blood flow (9,39,41). A distinguishing feature of intrahepatic portal hypertension (ex. liver cirrhosis), is persistent renal reabsorption of water and sodium, triggered to replenish the perceived central hypovolemia due to blood pooling in the periphery (9). Also evident in a more transient form in extrahepatic states of portal hypertension, fluid retention by the kidneys is secondary to a reduction in renal blood flow, consequently impairing renal function (9). A build-up of fluid in the splanchnic vasculature (from which the portal vein drains blood), secondary to hepatic blockage and renal salt and water retention, creates a pressure differential stimulating an increase in splanchnic and hepatic lymph flow (41); in essence, extravasation serves to relieve some of the excess fluid of the splanchnic bed. However, as the disease worsens this excess accumulates in the interstitium of the abdomen, leading to the formation of ascites (59,72). Consequently, portosystemic collaterals are formed to provide an outlet for excess fluid by increasing the capacity of the splanchnic vascular bed, but to no avail as portal pressure typically remains elevated (41,45,81).

There have been a number of hypotheses proposed to explain the "bipolar" nature of the hyperdynamic circulation observed in portal hypertension, such that an expanded plasma volume and fluid accumulation would lead to further salt and water retention (32,84). The most recently accepted theory, known as the *peripheral arterial vasodilator* theory, suggests that nitric oxide (NO)-mediated arteriolar vasodilation is the primary event leading to underfilling of the arterial circulation (72,79). Due to pooling in the periphery, central blood volume appears reduced, and this discordant signal triggers baroreceptor-mediated reflex retention of sodium and water by the kidneys (25,42). As evidence, treatment of portal hypertension with the introduction of a porto-caval anastomosis, and portal-systemic shunting in general has been associated with significant exacerbation of the systemic vasodilation occurring in portal hypertension. (15)

1.4.2. Renal and endocrine systems

The kidneys play a critical role in the etiology of portal hypertension. Renal sodium and water retention is caused by a decrease in sodium excretion (7,20). As portal hypertension worsens (ex. decompensated cirrhosis), glomerular filtration rate and renal blood flow also tend to fall (72). These changes are dependent on renal sympathetic nerve activity (RSNA), as evidenced by the fact that renal denervation completely attenuates this response (4). Activation of renal efferent nerves may also mediate endocrine abnormalities in portal hypertension, such as an increase in plasma renin activity (6,64), renal unresponsiveness to atrial natriuretic factor (ANF) (64,80), and/or enhanced vasopressin (AVP) secretion.

1.4.3. Nervous system

It is becoming increasingly apparent that CNS regulatory mechanisms are disordered in liver disease (1,17,35). A recent study implicates altered central cardiovascular regulation as a causal factor in developing the hyperdynamic circulation characteristic of cirrhosis (74). It is commonly reported that portal hypertension is characterized by a generalized increase in sympathetic outflow to the periphery (19,24,44). There is additional evidence which suggests a role for splanchnic sensory nerves in modulating the sympathetic nervous response in portal hypertension. In their studies examining the effect of neonatal capsaicin treatment on portal hypertensive and cirrhotic rats, Lee *et al.* found that intact primary afferents were required for the development of the hyperdynamic circulation, renal dysfunction and ascites formation in cirrhosis (55,57).

Possibilities as to the identity of such sensory afferents involved in the pathogenesis of portal hypertension include hepatic, mesenteric and/or splenic afferent nerves. Hepatic afferent nerve activity is increased in response to elevated intrahepatic pressure in cirrhosis (31,52). Accordingly, a reflex pathway with the kidney, known as the hepatorenal baroreflex, plays an important role in mediating renal dysfunction in the hepatorenal syndrome typical of advanced liver disease (31,39,54). Another possible player in mediating the portal hypertension-induced reflex renal response is the mesenteric afferent nerves, which would be stimulated by intestinal venous congestion (5). A final proposed participant in generating portal hypertension-induced renal dysfunction is splenic afferent nerve activity. As mentioned in the preceding section on splenic innervation, a proportion of fibers identified in the spleen are thought to be sensory and peptidergic (33,60,61); as well, reflex increases in renal sympathetic nerve activity had previously been demonstrated upon electrical stimulation of splenic afferent nerves (18,43).

1.5. THE SPLENORENAL REFLEX

The splenorenal reflex was discovered serendipitously during an investigation on the effects of NO on fluid extravasation from the spleen (30). It was found that selective inhibition of intrasplenic NO biosynthesis with a very small dose of the nitric oxide synthase inhibitor, NG-monomethyl-L-arginine (L-NMMA), increased systemic blood pressure (30). The same dose had no effect when administered systemically and furthermore, the observed response was abolished by either splenic or renal denervation, or by angiotensin-converting enzyme (ACE) inhibition (30). It was proposed that the spleen influences systemic blood pressure through a reflex pathway comprising splenic afferent nerves and renal sympathetic control of renin release (30).

Further investigation of the splenorenal reflex prompted a closer look at the role of splenic afferents in mediating portal hypertension-induced renal dysfunction (40,50). Using a model of portal hypertension, known as portal vein ligation (PVL), an increase in portal venous pressure was found to increase both splenic afferent and renal efferent nerve activity, confirming the existence of a splenorenal reflex (40,50). Moreover, partial occlusion of the portal vein caused a reflex reduction in renal blood flow, an effect which was abolished by renal or splenic denervation, or by phenoxybenzamine blockade of $\alpha 1$ adrenoreceptors (40). This finding confirmed the role of the splenorenal reflex in portal hypertension (Fig. 1-2).

Since it was known that portal hypertension not only alters intrahepatic hemodynamics (39,41) but also increases splenic venous outflow pressure (40), the isolated effect of the spleen in mediating reduced renal blood flow was examined. In these experiments, the effect of portal hypertension was mimicked using partial occlusion of the splenic vein (whereby splenic venous outflow pressure is increased without accompanying effects on the liver or mesentery), and the corresponding change in renal blood flow was observed. The same results were found; partial occlusion of the splenic vein caused an immediate reduction in renal blood flow, an effect inhibited by prior splenic or renal denervation, or $\alpha 1$ blockade of renal sympathetic nerve activity (40,41).

1.6. RATIONALE FOR EXPERIMENTS

The splenorenal reflex had been confirmed as the mediator of reduced renal blood flow in response to portal hypertension-induced changes in intrasplenic hemodynamics (40). However, there remained ambiguity in the natural stimulus for activating this reflex: partial occlusion of either the portal or splenic vein not only raises splenic venous pressure, but also reduces venous outflow from the spleen. The question therefore remained as to whether it was increased intrasplenic pressure and/or reduced intrasplenic blood flow which triggered increased splenic afferent nerve activity. The objective of the study presented in Chapter 2 was to distinguish between these possibilities.

An alteration in intrasplenic hemodynamics was known to affect systemic blood pressure through the splenorenal reflex, both in response to altered intrasplenic NO and secondary to portal hypertension (30,40). Although the splenorenal reflex had been previously identified to be a spinal neural reflex (67), the possibility remained that its effects were mediated in part through central neural pathways. Chapter 3 focuses on a study designed to determine whether splenic afferent nerve input influences central neural pathways involved in cardiovascular regulation, and if so, where such input is focused.

FIGURES

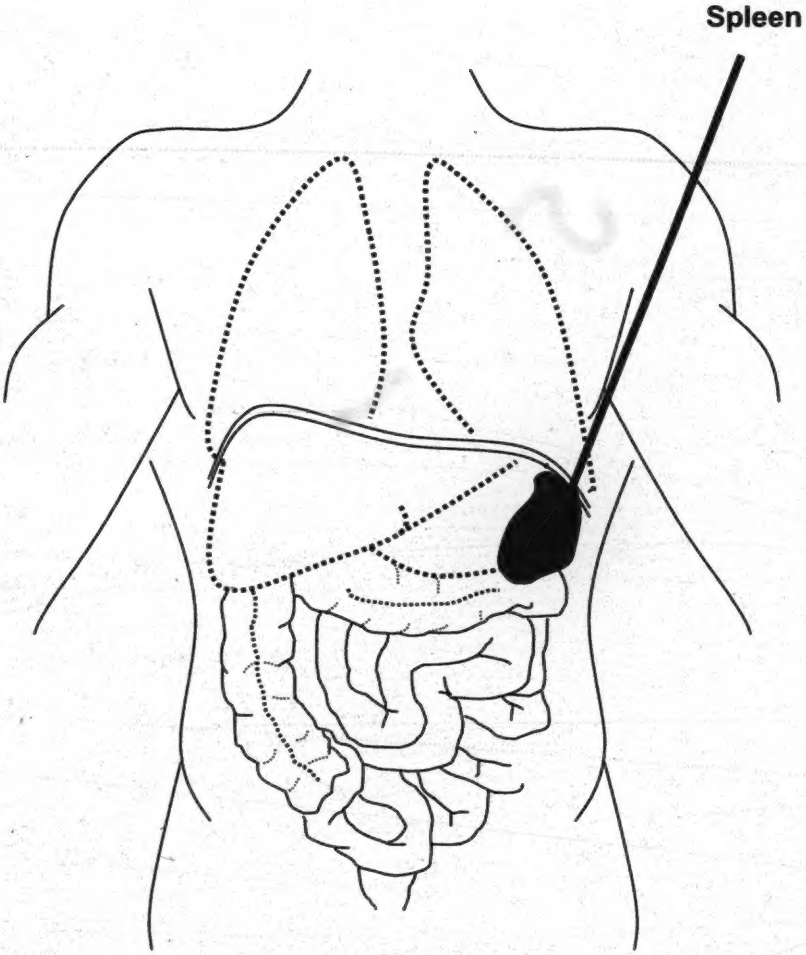


Figure 1-1. Location of the human spleen extending from the lower epigastric to upper lumbar regions of the thorax.

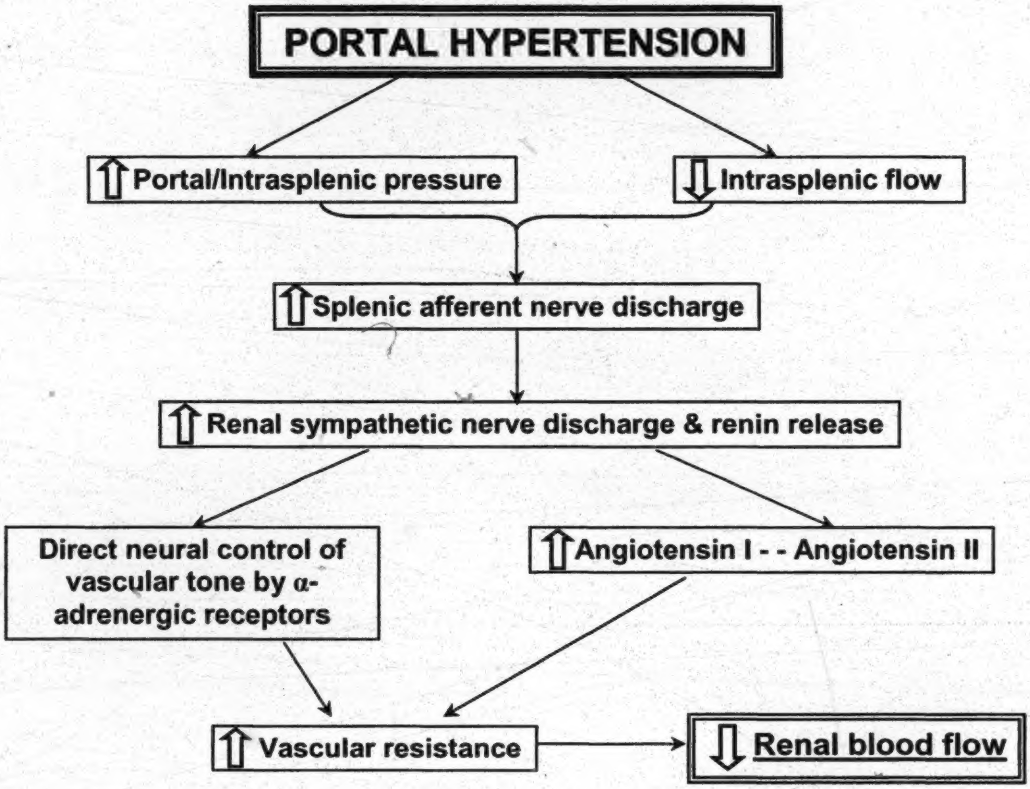

PORTAL HYPERTENSION
⇧ Portal/Intrasplenic pressure
⇩ Intrasplenic flow
⇧ Splenic afferent nerve discharge
⇧ Renal sympathetic nerve discharge & renin release
Direct neural control of vascular tone by α-adrenergic receptors
⇧ Angiotensin I - - Angiotensin II
⇧ Vascular resistance
⇩ Renal blood flow

Figure 1-2. Flowchart demonstrating the role of the splenorenal reflex pathway in exacerbating renal dysfunction in portal hypertension.

BIBLIOGRAPHY

1. Abergel, A., A. Braillon, C. Gaudin, G. Kleber, and D. Lebrec. Persistence of a hyperdynamic circulation in cirrhotic rats following removal of the sympathetic nervous system. *Gastroenterology* 102: 656-60, 1992.

2. Ackerman, K. D., S. Y. Felten, D. L. Bellinger, and D. L. Felten. Noradrenergic sympathetic innervation of the spleen: III. Development of innervation in the rat spleen. *J Neurosci Res* 18: 49-54, 123-5, 1987.

3. Ackerman, K. D., S. Y. Felten, C. D. Dijkstra, S. Livnat, and D. L. Felten. Parallel development of noradrenergic innervation and cellular compartmentation in the rat spleen. *Exp Neurol* 103: 239-55, 1989.

4. Anderson, R. J., R. E. Cronin, K. M. McDonald, and R. W. Schrier. Mechanisms of portal hypertension-induced alterations in renal hemodynamics, renal water excretion, and renin secretion. *J Clin Invest* 58: 964-70, 1976.

5. Andrews, C. J., W. H. Andrews, and J. Orbach. A sympathetic reflex elicited by distension of the mesenteric venous bed. *J Physiol* 226: 119-31, 1972.

6. Arroyo, V. and P. Gines. Mechanism of sodium retention and ascites formation in cirrhosis. *J Hepatol* 17: S24-8, 1993.

7. Atucha, N. M. and J. Garcia-Estan. Intrarenal alterations in experimental liver cirrhosis. *News in Physiological Sciences* 11: 48-52, 1996.

8. Baron, R. and W. Janig. Sympathetic and afferent neurons projecting in the splenic nerve of the cat. *Neurosci Lett* 94: 109-13, 1988.

9. Battarbee, H. D., J. H. Zavecz, and K. W. Betzing. Vagal and sympathetic components of the heart rate reflex in chronic portal vein stenosis. *Am J Physiol* 269:

G892-901, 1995.

10. Bellinger, D. L., S. Y. Felten, T. J. Collier, and D. L. Felten. Noradrenergic sympathetic innervation of the spleen: IV. Morphometric analysis in adult and aged F344 rats. *J Neurosci Res* 18: 55-63, 126-9, 1987.

11. Bellinger, D. L., S. Y. Felten, D. Lorton, and D. L. Felten. Origin of noradrenergic innervation of the spleen in rats. *Brain Behav Immun* 3: 291-311, 1989.

12. Bellinger, D. L., D. Lorton, R. W. Hamill, S. Y. Felten, and D. L. Felten. Acetylcholinesterase staining and choline acetyltransferase activity in the young adult rat spleen: lack of evidence for cholinergic innervation. *Brain Behav Immun* 7: 191-204, 1993.

13. Beluli, D. J. and L. C. Weaver. Areas of rostral medulla providing tonic control of renal and splenic nerves. *Am J Physiol* 261: H1687-92, 1991.

14. Beluli, D. J. and L. C. Weaver. Differential control of renal and splenic nerves without medullary topography. *Am J Physiol* 260: H1072-9, 1991.

15. Bernadich, C., J. C. Bandi, C. Piera, J. Bosch, and J. Rodes. Circulatory effects of graded diversion of portal blood flow to the systemic circulation in rats: role of nitric oxide. *Hepatology* 26: 262-7, 1997.

16. In Bowdler, A. J., ed. The spleen: Structure, function and clinical significance. London, Chapman and Hall Medical. 1990, xi-xvi.

17. Breitman, D. R. and S. S. Lee. Blunted responsiveness of the neuronal activation marker Fos in brainstem cardiovascular nuclei of cirrhotic rats. *Hepatology* 26: 1380-5, 1997.

18. Calaresu, F. R., J. C. Tobey, S. R. Heidemann, and L. C. Weaver. Splenic and renal

sympathetic responses to stimulation of splenic receptors in cats. *Am J Physiol* 247: R856-65, 1984.

19. Caramelo, C. , A. Fernandez-Cruz, L. M. Villamediana, E. Sanz, D. Rodriguez-Puyol, L. Hernando, and J. M. Lopez-Novoa. Systemic and regional haemodynamic effects of a synthetic atrial natriuretic peptide in conscious rats. *Clin Sci (Lond)* 71: 323-5.

20. Caregaro, L. , S. Lauro, P. Angeli, C. Merkel, and A. Gatta. Renal water and sodium handling in compensated liver cirrhosis: mechanism of the impaired natriuresis after saline loading. *Eur J Clin Invest* 15: 360-4, 1985.

21. Carlson, S. L., D. L. Felten, S. Livnat, and S. Y. Felten. Noradrenergic sympathetic innervation of the spleen: V. Acute drug-induced depletion of lymphocytes in the target fields of innervation results in redistribution of noradrenergic fibers but maintenance of compartmentation. *J Neurosci Res* 18: 64-9, 130-1, 1987.

22. Chen, A. and S. Kaufman. Splenic blood flow and fluid efflux from the intravascular space in the rat. *J Physiol* 490 (Pt 2): 493-9, 1996.

23. Chevendra, V. and L. C. Weaver. Distribution of splenic, mesenteric and renal neurons in sympathetic ganglia in rats. *J Auton Nerv Syst* 33: 47-54, 1991.

24. Claira, J., W. Jimenez, V. Arroyo, G. La Villa, C. Lopez, M. Asberta, A. Castro, J. Gaya, F. Rivera, and J. Rodes. Effect of V_1- vasopressin receptor blockade on arterial pressure in conscious rats with cirrhosis and ascites. *Gastroenterology* 100: 494-501, 1991.

25. Colombato, L. A., A. Albillos, and R. J. Groszmann. The role of central blood volume in the development of sodium retention in portal hypertensive rats. *Gastroenterology* 110: 193-8, 1996.

26. Cooper, M. J. and R. C. Williamson. Splenectomy: indications, hazards and

alternatives. *Br J Surg* 71: 173-80, 1984.

27. Crosby, W. H. An historical sketch of splenic function and splenectomy. *Lymphology* 16: 52-5, 1983.

28. Davies, B. N. and P. G. Withrington. The actions of drugs on the smooth muscle of the capsule and blood vessels of the spleen. *Pharmacol Rev* 25: 373-412, 1973.

29. Deng, Y., L. Copeman, and S. Kaufman. A novel natriuretic, hypotensive factor derived from the spleen. *Life Sci* 57: 1647-53, 1995.

30. Deng, Y. and S. Kaufman. Splenorenal reflex regulation of arterial pressure. *Hypertension* 38: 348-52, 2001.

31. DiBona, G. F. Renal neural activity in hepatorenal syndrome. *Kidney Int* 25: 841-53, 1984.

32. Dunbar, B. S., J. R. Elk, R. E. Drake, and G. A. Laine . Intestinal lymphatic flow during portal venous hypertension. *Am J Physiol* 257: G94-8, 1989.

33. Elfvin, L. G., H. Aldskogius, and J. Johansson. Splenic primary sensory afferents in the guinea pig demonstrated with anterogradely transported wheat-germ agglutinin conjugated to horseradish peroxidase. *Cell Tissue Res* 269: 229-34, 1992.

34. Ermirio, R., P. Ruggeri, C. Molinari, and L. C. Weaver. Somatic and visceral inputs to neurons of the rostral ventrolateral medulla. *Am J Physiol* 265: R35-40, 1993.

35. Fernandez, B., M. Vatta, M. Rodriguez-Fermepin, L. Bianciotti, S. Romay, F. Eizayaga, J. Perazzo, and A. Lemberg. Norepinephrine uptake modifications in circumventricular organs, pons, and myelencephalic areas and nuclei in prehepatic portal hypertensive rats. *Neurochem Int* 34: 177-81, 1999.

36. Greenway, C. V. Role of splanchnic venous system in overall cardiovascular

homeostasis. *Fed Proc* 42: 1678-84, 1983.

37. In Groom, A. C. and E. E. Schmidt, eds. Chapter 4: Microcirculatory blood flow through the spleen. London, Chapman and Hall Medical. 1990, 45-102.

38. Groom, A. C. and S. H. Song. Effects of norepinephrine on washout of red cells from the spleen. *Am J Physiol* 221: 255-8, 1971.

39. Gupta, T. K. , L. Chen, and R. J. Groszmann. Pathophysiology of portal hypertension. *Baillieres Clin Gastroenterol* 11: 203-19, 1997.

40. Hamza, S. M. and S. Kaufman. Splenorenal reflex modulates renal blood flow in the rat. *J Physiol* 558: 277-82, 2004.

41. Henderson, J. M., D. S. Barnes, and M. A. Geisinger. Portal Hypertension. *Curr Probl Surg* 35: 379-452, 1998.

42. Henriksen, J. H., F. Bendtsen, T. I. A. Sorensen, C. Stadeager, and H. Ringlarsen. Reduced central blood volume in cirrhosis. *Gastroenterology* 97: 1506-13, 1989.

43. Herman, N. L., D. R. Kostreva, and J. P. Kampine. Splenic afferents and some of their reflex responses. *Am J Physiol* 242: R247-54, 1982.

44. Joh, T., D. N. Granger, and J. N. Benoit. Endogenous vasoconstrictor tone in intestine of normal and portal hypertensive rats. *Am J Physiol* 264: H171-7, 1993.

45. Kaji, T., J. Tamura, M. Matsumoto, K. Hoshino, T. Takeuchi, S. Sato, H. Murakami, S. Yano, and T. Naruse. Superior mesenteric varices and splenic renal shunt with portal hypertension - a case report of liver cirrhosis with complications. *J Int Med Res* 21: 58-65, 1993.

46. Kaufman, S. Role of spleen in ANF-induced reduction in plasma volume. *Can J Physiol Pharmacol* 70: 1104-8, 1992.

47. Kaufman, S. and Y. Deng. Splenic control of intravascular volume in the rat. *J Physiol* 468: 557-65, 1993.

48. Kaufman, S. and Y. Deng. Control of systemic blood pressure: influence of splenic afferent nerves and nitric oxide (NO). *The FASEB Journal* 13: A458, 1999.

49. Kaufman, S. and Y. Deng. Effect of splenic extract on plasma volume and renal function in the rat. *Life Sci* 65: 2653-62, 1999.

50. Kaufman, S. and J. Levasseur. Effect of portal hypertension on splenic blood flow, intrasplenic extravasation and systemic blood pressure. *Am J Physiol Regul Integr Comp Physiol* 284: R1580-5, 2003.

51. Klein, R. L., S. P. Wilson, D. J. Dzielak, W. H. Yang, and O. H. Viveros. Opioid peptides and noradrenaline co-exist in large dense-cored vesicles from sympathetic nerve. *Neuroscience* 7: 2255-61, 1982.

52. Kostreva, D. R., A. Castaner, and J. P. Kampine. Reflex effects of hepatic baroreceptors on renal and cardiac sympathetic nerve activity. *Am J Physiol* 238: R390-4, 1980.

53. Koyama, S., F. Sawano, Y. Matsuda, Y. Saeki, T. Shibamoto, T. Hayashi Jr, Y. Matsubayashi, and M. Kawamoto. Spatial and temporal differing control of sympathetic activities during hemorrhage. *Am J Physiol* 262: R579-85, 1992.

54. Lang, F., W. Gerok, and D. Haussinger. New clues to the pathophysiology of hepatorenal failure. *Clin Investig* 71: 93-7, 1993.

55. Lee, S. S. and K. A. Sharkey. Capsaicin treatment blocks development of hyperkinetic circulation in portal hypertensive and cirrhotic rats. *Am J Physiol* 264: G868-73, 1993.

56. Levesque, M. J. and A. C. Groom. Fast transit of red cells and plasma in contracted

versus relaxed spleens. *Can J Physiol Pharmacol* 59: 53-8, 1981.

57. Li, Y., D. Song, Y. Zhang, and S. S. Lee. Effect of neonatal capsaicin treatment on haemodynamics and renal function in cirrhotic rats. *Gut* 52: 293-9, 2003.

58. Lockwood, C. M. Immunological functions of the spleen. *Clin Haematol* 12: 449-65, 1983.

59. Longmire-Cook, S. J. Pathophysiologic factors and management of ascites. *Surg Gynecol Obstet* 176: 191-202, 1993.

60. Lorton, D., D. L. Bellinger, S. Y. Felten, and D. L. Felten. Substance P innervation of spleen in rats: nerve fibers associate with lymphocytes and macrophages in specific compartments of the spleen. *Brain Behav Immun* 5: 29-40, 1991.

61. Lundberg, J. M., A. Anggard, J. Pernow, and T. Hokfelt. Neuropeptide Y-, substance P- and VIP-immunoreactive nerves in cat spleen in relation to autonomic vascular and volume control. *Cell Tissue Res* 239: 9-18, 1985.

62. Lundgren, O. Role of splanchnic resistance vessels in overall cardiovascular homeostasis. *Fed Proc* 42: 1673-7, 1983.

63. Maass-Moreno, R. and C. F. Rothe. Carotid baroreceptor control of liver and spleen volume in cats. *Am J Physiol* 260: H254-9, 1991.

64. Martin, P. Y. and R. W. Schrier. Pathogenesis of water and sodium retention in cirrhosis. *Kidney Int Suppl* 51: S43-9, 1997.

65. McClusky, D. A. 3rd, L. J. Skandalakis, G. L. Colborn, and J. E. Skandalakis. Tribute to a triad: history of splenic anatomy, physiology, and surgery--part 1. *World J Surg* 23: 311-25, 1999.

66. Meckler, R. L. and L. C. Weaver. Characteristics of ongoing and reflex discharge of

single splenic and renal sympathetic postganglionic fibres in cats. *J Physiol* 396: 139-53, 1988.

67. Meckler, R. L. and L. C. Weaver. Persistent firing of splenic and renal afferent nerves after acute decentralization but failure to produce ganglionic reflexes. *Neurosci Lett* 88: 167-72, 1988.

68. Mignini, F., V. Streccioni, and F. Amenta. Autonomic innervation of immune organs and neuroimmune modulation. *Auton Autacoid Pharmacol* 23: 1-25, 2003.

69. Nance, D. M. and J. Burns. Innervation of the spleen in the rat: Evidence for absence of afferent innervation. *Brain Behav Immun* 3: 281-90, 1989.

70. Reilly, F. D. Innervation and vascular pharmacodynamics of the mammalian spleen. *Experientia* 41: 187-92, 1985.

71. Rogausch, H., A. D. Rey, A. Kabiersch, and H. O. Besedovsky. Interleukin-1 increases splenic blood flow by affecting the sympathetic vasoconstrictor tonus. *Am J Physiol* 268: 902-8, 1995.

72. Schrier, R. W., V. Arroyo, M. Bernardi, M. Epstein, J. H. Henriksen, and J. Rodes. Peripheral arterial vasodilation hypothesis: a proposal for the initiation of renal sodium and water retention in cirrhosis. *Hepatology* 8: 1151-7, 1988.

73. Shoukas, A. A., C. L. MacAnespie, M. J. Brunner, and L. Watermeier. The importance of the spleen in blood volume shifts of the systemic vascular bed caused by the carotid sinus baroreceptor reflex in the dog. *Circ Res* 49: 759-66, 1981.

74. Song, D., H. Liu, K. A. Sharkey, and S. S. Lee. Hyperdynamic circulation in portal-hypertensive rats is dependent on central c-fos gene expression. *Hepatology* 35: 159-66, 2002.

75. Spencer, R. P. and H. A. Pearson. The spleen as a hematological organ. *Semin Nucl Med* 5: 95-102, 1975.

76. Stewart, I. B., D. E. Warburton, A. N. Hodges, D. M. Lyster, and D. C. McKenzie. Cardiovascular and splenic responses to exercise in humans. *J Appl Physiol* 94: 1619-26, 2003.

77. Stock, R. J., E. V. Cilento, F. D. Reilly, and R. S. McCuskey. A compartmental analysis of the splenic circulation in rat. *Am J Physiol* 245: H17-21, 1983.

78. Straub, R. H. Complexity of the bi-directional neuroimmune junction in the spleen. *Trends Pharmacol Sci* 25: 640-6, 2004.

79. Vallance, P. and S. Moncada. Hyperdynamic circulation in cirrhosis: a role for nitric oxide? *Lancet* 337: 776-8, 1991.

80. Warner, L. C., W. M. Leung, P. Campell, J. Miller, A. Logan, L. M. Blendis, and K. L. Skorecki. The role of resistance to atrial natriuretic peptide in the pathogenesis of sodium retention in hepatic cirrhosis. *Progress in Atrial Peptide Research* 3: 185-204, 1989.

81. Wattanasirichaigoon, S., F. D. Gordon, and R. H. Resnick. Hyperdynamic circulation in portal hypertension: a comparative model of arterio-venous fistula. *Med Hypotheses* 55: 77-87, 2000.

82. Weaver, L. C., H. K. Fry, and R. L. Meckler. Differential renal and splenic nerve responses to vagal and spinal afferent inputs. *Am J Physiol* 246: R78-87, 1984.

83. In Weiss, L., ed. Chapter 3: Mechanisms of splenic clearance of the blood; a structural overview of the mammalian spleen. London, Chapman and Hall Medical. 1990, 23-43.

84. Witte, M. H., C. L. Witte, and A. E. Dumont. Progress in liver disease: Physiological factors involved in the causation of cirrhotic ascites. *Gastroenterology* 61: 742-50, 1971.

CHAPTER 2. SPLENIC BARORECEPTORS CONTROL SPLENIC AFFERENT NERVE ACTIVITY

A version of this chapter has been published.

Moncrief & Kaufman 2006. Am J Physiol Regul Integr Comp Physiol 290: R352-6

2.1. INTRODUCTION

The spleen contributes to homeostatic regulation of both blood volume and pressure. It is a major site of regulated fluid efflux out of the vasculature and into the extravascular fluid compartment (5,7). In addition, structural and functional evidence has pointed to the existence of a neural pathway between the spleen and kidneys, through which the spleen may influence renal function (6,16,24). In addition, we have shown there to be an inverse relationship between intrasplenic nitric oxide (NO) and mean arterial pressure (MAP) (9), a response which is abolished by either splenic or renal denervation. This led us to propose that intrasplenic hemodynamics might initiate a splenorenal reflex.

Further evidence for such a reflex arose from studies examining the effects of hepatic portal hypertension on splenic and renal hemodynamics. Perturbations of renal hemodynamics are an important pathological consequence of portal hypertension, and it has long been established that the hepatorenal reflex contributes to this dysfunction (17). However, in addition to altering intrahepatic hemodynamics (13,15), portal hypertension also increases splenic venous outflow pressure (14). We showed that controlled partial occlusion of either the portal or the splenic vein increases both splenic afferent nerve activity and renal efferent nerve activity (14,19). Thus we proposed that, in addition to the hepatorenal reflex, a splenorenal reflex also contributes to portal hypertension-induced renal dysfunction.

Partial occlusion of either the portal or the splenic vein not only raises splenic venous pressure but also reduces venous outflow from the spleen. The question thus remained as to whether it was increased intrasplenic post-capillary pressure and/or reduced intrasplenic blood flow that triggers the increase in splenic afferent nerve activity. The objective of this study was to distinguish between these possibilities. We compared the relative effects on splenic afferent nerve activity of *1)* partial occlusion of the splenic vein, which selectively decreased splenic arterial and venous blood flow and *increased* splenic venous pressure, and *2)* partial occlusion of the splenic artery, which similarly decreased splenic arterial and venous blood flow, but caused no change in splenic venous pressure. We were thus able to eliminate flow as a variable when we selectively raised splenic venous pressure.

2.2. METHODS

All experimental procedures were approved by the local Animal Welfare Committee according to the guidelines set by the Canada Council on Animal Care. Upon completion of each experiment, all animals were euthanized with an anesthetic overdose of pentobarbital sodium (96 mg iv, MTC Pharmaceuticals, Cambridge, ON).

2.2.1. Animals and housing

Male Long Evans rats were purchased from Charles River Canada (St. Foy, QB, Canada) and housed in the University of Alberta Animal Facility in a temperature-controlled environment, exposed to light on a 12:12-h light-dark cycle, and given water ad libitum. They were fed a restricted diet of 0.3% sodium rat chow (three pellets daily) for 2 wk before experiments were started, so as to reduce intraabdominal fat (20). There were three experimental groups: splenic vein-occluded rats (SVO, n = 6), and two groups of splenic artery-occluded (SAO) rats comprising animals in which arterial blood flow was matched to that observed in the SVO group (arterial flow-matched splenic artery occlusion, SAO_{AF}, n = 6), and animals in which venous blood flow was matched to that observed in the SVO group (venous flow-matched splenic artery occlusion, SAO_{VF}, n = 5).

2.2.2. Surgery

Anesthesia was induced with sodium pentobarbital (62 mg/kg body wt, ip) and maintained with Inactin (thiobutabarbital sodium, 80 mg/kg body wt, sc, Sigma, St. Louis, MO). Temperature was maintained using Delta-phase heating pads (Braintree Scientific, Braintree, MA).

The femoral vein and artery were cannulated with Silastic tubing (0.51 mm ID, 0.94 mm OD; Dow Corning Corporation, Midland, MI) and polyethylene tubing (PE-50, 0.58 mm ID, 0.97 mm OD; Becton Dickinson, Sparks, MD) for infusion of isotonic saline (3 ml/h) and measurement of systemic blood pressure, respectively. A midline laparotomy was performed to expose the spleen and its vasculature (Fig. 2-1). Silastic tubing (0.30 mm ID, 0.64 mm OD) was inserted occlusively into the gastric vein, and its tip advanced to the junction with the splenic vein. Similarly, polyethylene tubing (PE-10, 0.28 mm ID, 0.61 mm OD) was inserted into the gastric artery and advanced to the junction with the splenic artery. Cannulas were connected to Gould Statham pressure transducers (Gould Electronics, Recording Systems Division, Cleveland, OH) for online measurement of splenic venous and arterial pressure. The splenic vasculature was isolated by ligating all vessels leading to or from other vascular beds (L1/L2; Fig. 2-1). A cavity was created by securing the edges of the abdominal incision to a wire support ring to contain the warm mineral oil needed to electrically isolate the nerve.

2.2.3. Splenic vein occlusion

A balloon occluder was placed around the splenic vein proximal to the junction with the gastric vein; this could be inflated to allow for controlled, partial occlusion of the vessel (splenic vein occlusion, SVO; Fig. 2-1). Factory-calibrated transit-time flow probes (0.5V and 1RB series, Transonic Systems, Ithaca, NY) were placed around the splenic artery (0.5V) and splenic vein (1RB), distal to the junction with the gastric vessels but proximal to the point of branching of the smaller splenic vessels.

2.2.4. Arterial flow-matched and venous flow-matched splenic artery occlusions

Surgical procedures for the arterial occlusion groups were similar to those for splenic vein occlusion, except that *1)* the balloon occluder was positioned around the splenic artery distal to the junction with the gastric, celiac and hepatic arteries (SAO; Fig. 2-1); *2)* the gastric artery was occluded, but not cannulated (the placement of the occluder was such that we were unable to obtain downstream arterial pressure measurements in these animals); and *3)* single flow probes were positioned around the vein or artery, for the venous flow-matched group or the arterial flow-matched group respectively.

2.2.5. Extracellular nerve recording

The splenic nerve is located as a bundle between the splenic artery and vein (Fig. 2-1). Under mineral oil, a section of the splenic nerve was carefully isolated, divided, and sectioned for recording of splenic sensory afferent nerve activity. The distal end of the cut nerve (coming from the spleen) was laid on a bipolar platinum recording electrode. The signals were amplified using an isolation preamplifier (model 11-5407-58) and universal amplifier (model 13-4615-58, Gould Electronics). Nerve activity was recorded online at a sample rate of 10,000 Hz, amplified and filtered between 100 and 10,000 Hz.

2.2.6. Experimental protocol

After completion of all surgical procedures, animals were allowed to stabilize for ~30 minutes. Selection criteria were used to standardize our experimental groups. Inclusion was contingent on achieving baseline values of mean arterial pressure (MAP; 80-105 mmHg), splenic arterial blood flow (1.5-2.5 ml/min), splenic venous blood flow (1.0-2.5 ml/min), splenic venous pressure (SVP; 6-15 mmHg), and splenic afferent nerve activity (SANA; 1-4 spikes/second). Baseline values were recorded for 5 minutes before occlusion. In SVO animals, the balloon occluder was then inflated to increase splenic venous pressure to 20-24 mmHg for 5 min. This value was chosen according to a previously established protocol for partial splenic vein occlusion, mimicking the splenic venous pressure observed in portal hypertension (14). In the arterial flow-matched SAO animals, the balloon was inflated for 5 minutes so as to match the reduction in splenic arterial blood flow observed with SVO. In venous flow-matched SAO animals, the balloon was inflated for 5 minutes so as to match the reduction in splenic venous blood flow observed with SVO. The balloon was inflated gradually over a period of ~1 minute. For both SVO and SAO groups, occlusion values are the average of a 2-min period, beginning 1 min after the initiation of vessel occlusion.

2.2.7. Data analysis

Data are presented as mean ± SE. All data were recorded and analyzed using WINDAQ/Pro software (DATAQ Instruments, Akron, OH). Nerve activity was quantified as an average discharge rate (spikes/s) based on the visual identification of action potentials in the raw filtered recordings. Compound action potentials could be easily identified amongst the background noise, appearing as a distinct broadening of the electrical activity and increased magnitude above background noise. Nerve activity is represented as an average of each 5-min period for baseline, occluded and recovery data, with recovery values taken 5 min after the end of occlusion. Data were analyzed for multiple comparisons between groups using two-way repeated-measures analysis of variance (ANOVA), followed by the Student-Newman-Keuls method for post hoc analysis. Statistical significance was accepted at $P < 0.05$.

2.3. RESULTS

Comparisons of baseline values (before vessel occlusion) revealed no significant differences among any of the experimental groups with respect to systemic blood pressure, splenic venous pressure, splenic arterial and venous blood flows, or splenic afferent nerve activity (Table 2-1).

2.3.1. Effects of venous and arterial occlusion on splenic venous pressure, splenic arterial pressure and MAP

SVO and SAO had differing effects on splenic venous and arterial pressures. Splenic venous pressure increased during inflation of the balloon occluder around the splenic vein (SVO) but returned to baseline after release (Fig. 2-2), although there were no changes in splenic arterial pressure (baseline = 87.7 ± 1.6 mmHg, occlusion = 85.0 ± 4.4 mmHg, release = 86.3 ± 3.3 mmHg, $n = 6$; $P > 0.05$) or MAP (baseline = 90.2 ± 2.9 mmHg, occlusion = 86.1 ± 5.1 mmHg, release = 87.8 ± 4.2 mmHg, $n = 6$, $P > 0.05$). Splenic venous pressure did not change after partial occlusion or release of the splenic artery (Fig. 2-2; arterial flow-matched, $\mathrm{SAO_{AF}}$; venous flow-matched, $\mathrm{SAO_{VF}}$). Nor were there any significant changes in MAP ($\mathrm{SAO_{AF}}$: baseline = 90.7 ± 2.9, occlusion = 91.8 ± 2.2 mmHg, release = 90.9 ± 3.5 mmHg, $n = 6$, $P > 0.05$; $\mathrm{SAO_{VF}}$: baseline = 96.1 ± 1.2 mmHg, occlusion = 98.3 ± 2.2 mmHg, release = 100.5 ± 2.9 mmHg, $n = 5$, $P > 0.05$).

2.3.2. Matched reduction in splenic arterial and venous blood flow

After splenic vein occlusion, splenic arterial blood flow fell from 1.7 ± 0.1 to 0.9 ± 0.1 ml/min ($n = 6$, $P < 0.05$). Using these figures as our criteria for the arterial flow-matched SAO group, we partially occluded the splenic artery so as to match the fall in arterial flow (Fig. 2-3*A*). After splenic vein occlusion, splenic venous blood flow fell from 1.3 ± 0.1 to 0.6 ± 0.1 ml/min ($n = 6$). Similarly, using these values as our criteria for the venous flow-matched SAO group, we partially occluded the splenic artery so as to match the fall in venous flow (Fig. 2-3*B*). In all three groups, splenic arterial and venous flows recovered to baseline levels upon deflation of the balloon occluder (Fig. 2-3, *A* and *B*).

2.3.3. Differential effects of SVO and SAO on splenic afferent nerve activity

Partial occlusion of the splenic vein caused a marked increase in SANA (Fig. 2-4, SVO). In contrast, partial occlusion of the splenic artery did not cause any significant change in SANA (Fig. 2-4, SAO_{AF} and SAO_{VF}). In all three groups, SANA returned to baseline after deflating the balloon occluder. Furthermore, SANA correlated linearly with increases in splenic venous pressure ($r = 0.623$, $P = 0.008$, $n = 17$, Fig. 2-5). There was no such relationship between SANA and either splenic arterial blood flow ($r = 0.371$, $P = 0.236$, $n = 12$) or splenic venous blood flow ($r = 0.275$, $P = 0.413$, $n = 11$).

2.4. DISCUSSION

Partial occlusion of either the portal or splenic vein causes an immediate increase in splenic venous pressure accompanied by a corresponding fall in splenic blood flow, as well as an increase in splenic afferent nerve activity (14,19). Our previous finding that intrasplenic NO reflexly alters MAP (9) had raised the possibility that flow-mediated NO release, not pressure, might be the stimulus for splenic afferent nerve activity. To ascertain whether changes in intrasplenic post-capillary pressure or splenic blood flow stimulate afferent nerve activity, we designed the current experiments to eliminate flow as a variable, while we selectively raised splenic venous pressure. We found that partial occlusion of the splenic vein, which was associated with increased splenic venous pressure and decreased splenic blood flow, caused a 27% increase in splenic afferent nerve activity from baseline. By contrast, partial occlusion of the splenic artery, which was accompanied by the same fall in splenic blood flow but which caused no change in splenic venous pressure, had no effect on splenic afferent nerve activity. Regression analysis revealed a positive linear correlation between venous pressure and afferent nerve activity. There was no such relationship between blood flow and nerve activity. Higher splenic arterial flow than venous blood flow has previously been reported and has been ascribed to intrasplenic fluid extravasation (7).

Studies of the spleen have largely focused on its role in immune function. Furthermore, most studies regarding innervation of the spleen and its vasculature have focused on efferent sympathetic innervation and its role in modulating the immune system (2). A substantial proportion (~95%) of nerve fibers in the splenic nerve is sympathetic, most of which originate in the celiac-superior mesenteric ganglion complex (4). Indeed, the very existence of afferent innervation of the spleen had even at one time been questioned, although the authors of one study using fluorescent retrograde tracing did concede that there may be a small number of afferent fibers innervating the hilar region of the spleen and/or the associated vasculature (27). Functionally, a number of studies have clearly demonstrated that afferent fibers do convey sensory information from the spleen (11,12,16,24). Furthermore, evidence for afferent innervation of the spleen has now been demonstrated both histologically (11), and by direct electrophysiological measurement (19).

Our results suggest that intrasplenic post-capillary vascular pressure is monitored and controls splenic afferent nerve activity. This is consistent with findings that there are, in the dog and the cat (6,16), afferent nerve endings located in the wall of the splenic vein; that is, that there are splenic mechanoreceptors, which respond to alterations in venous wall tension. It has long been established that the hepatorenal and renorenal reflexes are sensed and initiated by intrahepatic and intrarenal mechanoreceptors, respectively (1,10,21,22,25,28). We propose that a similar mechanism regulates afferent neural discharge rate in the spleen.

Baroreceptors and stretch receptors are present throughout the cardiovascular system, providing regulatory feedback for the maintenance of systemic blood pressure and volume (23). We have previously observed that denervation of the spleen causes a progressive decline in MAP (9). This suggests that the spleen may play a role in the tonic maintenance of blood pressure under normal physiological conditions. In addition, chemical or mechanical stimulation of splenic afferent receptors has been shown to elicit reflex changes in cardiopulmonary sympathetic activity, leading to increased heart rate, contractile force, and systemic blood pressure (6,16). We have now shown that increased intrasplenic post-capillary pressure initiates an increase in splenic afferent nerve activity. This lends weight to our suggestion that the spleen, like the liver and the kidneys, should be considered a supplementary reflexogenic area involved in normal cardiovascular homeostasis.

2.4.1. Perspectives

Our data suggest that, in pathological conditions such as portal hypertension, increased splenic venous pressure would stimulate splenic venous sensory receptors and increase splenic afferent nerve activity. It is tempting to suggest that such a splenic response could serve as a compensatory mechanism to offset the systemic hypotension seen in both experimental and clinical portal hypertension (3,26). However, partial occlusion of the splenic or portal vein has previously been shown to cause a significant *reduction* in mean arterial pressure (14). This portal hypertension-induced fall in systemic pressure stands to worsen the condition by contributing to the already compromised renal and cardiovascular function. It is unlikely to be mediated by our observed increase in splenic afferent nerve activity, since such an increase would be expected to increase renin release (14,18). Furthermore, we have observed that the fall in MAP observed during splenic vein occlusion persists even after splenic denervation (14). (In these current studies, MAP did tend to fall during SVO but failed to reach significance when baseline, occlusion, and release were analyzed by repeated-measures ANOVA.) We suggest, therefore, that the systemic hypotension associated with splenic and/or portal hypertension is likely not neurally-mediated and may instead be caused by the release of an as-yet unidentified hypotensive factor from the spleen (8,14).

In summary, we have shown that a rise in splenic venous pressure, not a fall in splenic blood flow, is responsible for increasing splenic afferent nerve activity after obstruction of splenic venous outflow. The evidence points to this being mediated through sensory mechanoreceptors located in the walls of the splenic postcapillary vasculature. This study thus establishes that the spleen plays a significant role in cardiovascular regulation by monitoring splanchnic (venous) pressure. There is evidence that splenic blood flow influences splenic afferent nerve activity. We suggest that, in portal hypertension, it is the increased splenic venous outflow pressure, but not the venous stagnation which initiates an increase in splenic afferent nerve firing, activates the splenorenal reflex, and reduces renal blood flow.

2.5. GRANTS

This study was supported by grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Alberta, Northwest Territories, and Nunavut, Canada.

FIGURES

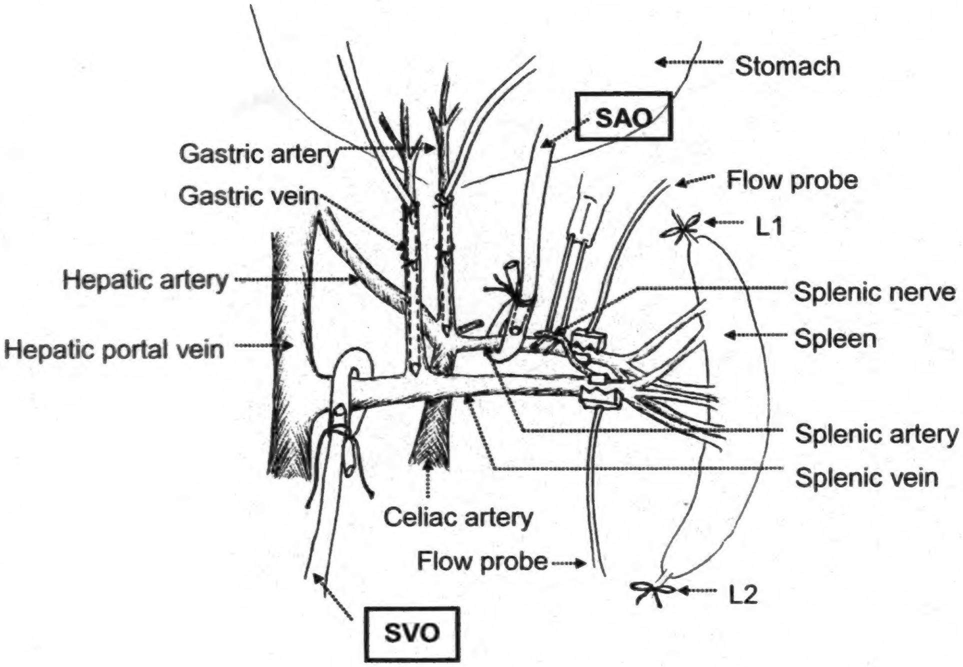

Stomach
SAO
Gastric artery
Gastric vein
Flow probe
L1
Hepatic artery
Splenic nerve
Spleen
Hepatic portal vein
Splenic artery
Splenic vein
Celiac artery
Flow probe
L2
SVO

Figure 2-1. Anatomy of the splenic circulation and configuration of balloon occluders, cannulas, flow probes and recording electrode. SVO, splenic vein occluder; SAO, splenic artery occluder; L1/L2, vascular isolation ligatures.

Table 2-1. Baseline values from SVO, venous flow-matched and arterial flow-matched SAO groups

	SVO	SAO_{VF}	SAO_{AF}
n	6	5	6
MAP, mmHg	90.2 ± 2.9	96.1 ± 1.2	90.7 ± 2.9
SABF, ml/min	1.7 ± 0.1		1.7 ± 0.1
SVBF, ml/min	1.3 ± 0.1	1.2 ± 0.1	
SVP, mmHg	9.5 ± 0.5	8.8 ± 1.1	8.3 ± 0.9
SANA, spikes/s	1.7 ± 0.4	1.9 ± 0.4	1.5 ± 0.1

Values are presented as means ± SE. SVO, splenic vein occlusion; SAO_{VF}, venous flow-matched splenic artery occlusion; SAO_{AF}, arterial flow-matched splenic artery occlusion; MAP, mean arterial pressure; SABF, splenic arterial blood flow; SVBF, splenic venous blood flow; SVP, splenic venous pressure; SANA, splenic afferent nerve activity; *n* = number.

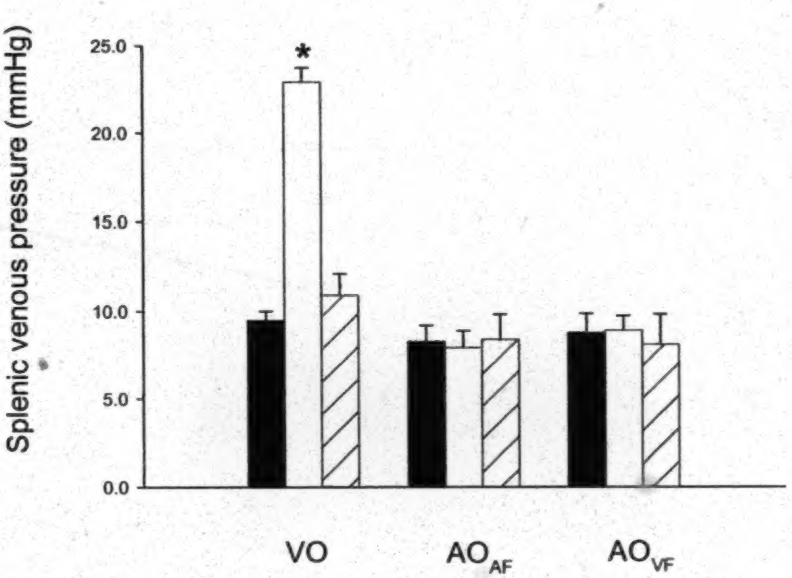


Figure 2-2. Effects of partial occlusion of the splenic vein (SVO, $n = 6$) or artery (SAO_{AF}, $n = 6$; SAO_{VF}, $n = 5$) on splenic venous pressure. Baseline (solid bars); partial occlusion (open bars); recovery (hatched bars). Vertical bars delineate SE of mean. *Significant difference from baseline, $P < 0.05$.

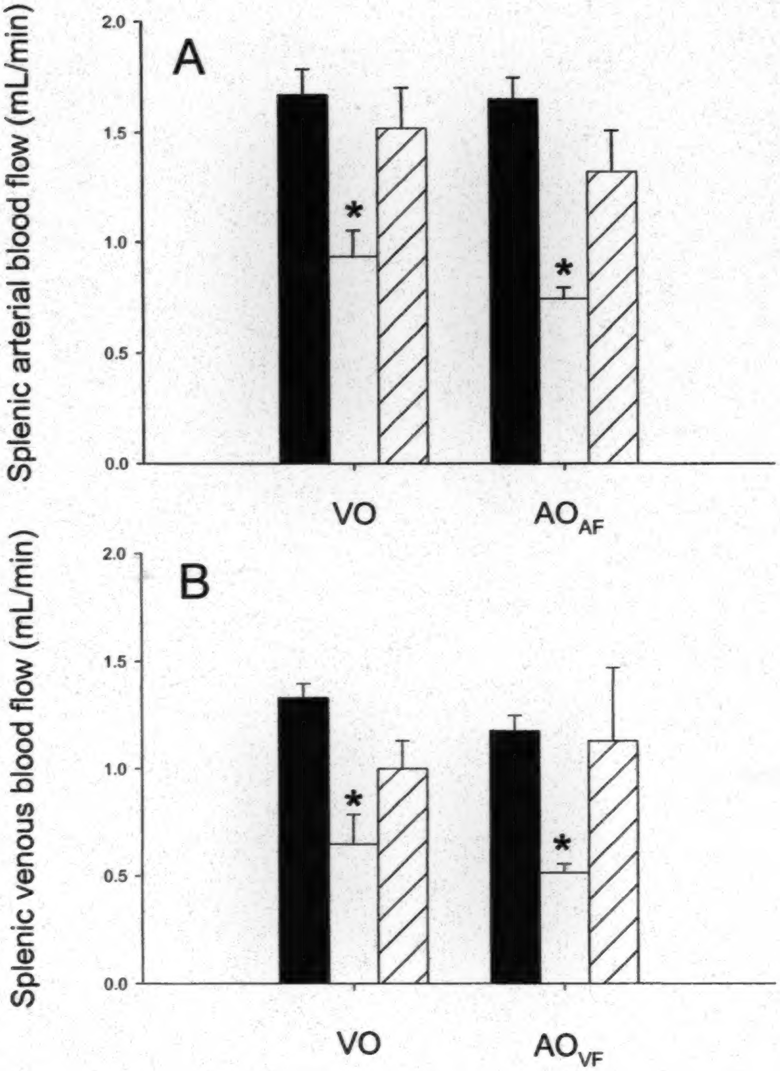


Figure 2-3. Effects of partial occlusion of the splenic vein (SVO, $n = 6$) or artery (SAO$_{AF}$, $n = 6$; SAO$_{VF}$, $n = 5$) on splenic arterial (*A*) and venous (*B*) blood flow. Baseline (solid bars); partial occlusion (open bars); recovery (hatched bars). Vertical bars delineate SE of mean. *Significant difference from baseline, $P < 0.05$.

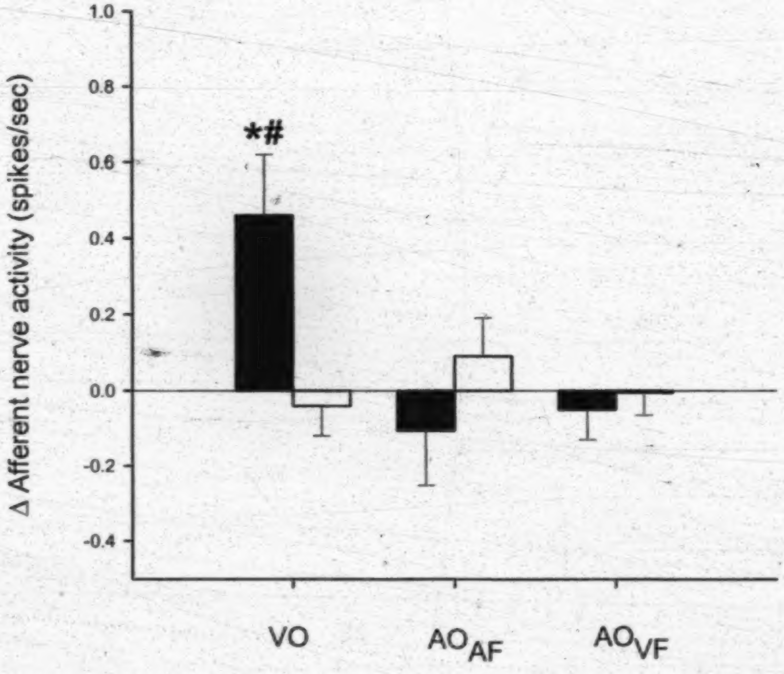


Figure 2-4. Effects of partial occlusion (solid bars) and release (open bars) of the splenic vein (SVO, $n = 6$) and artery (SAO_{AF}, $n = 6$ and SAO_{VF}, $n = 5$) on splenic afferent nerve activity. Vertical bars delineate SE of the mean. *Significant difference in afferent discharge rate from baseline; #significant difference from both artery-occluded groups, $P < 0.05$.

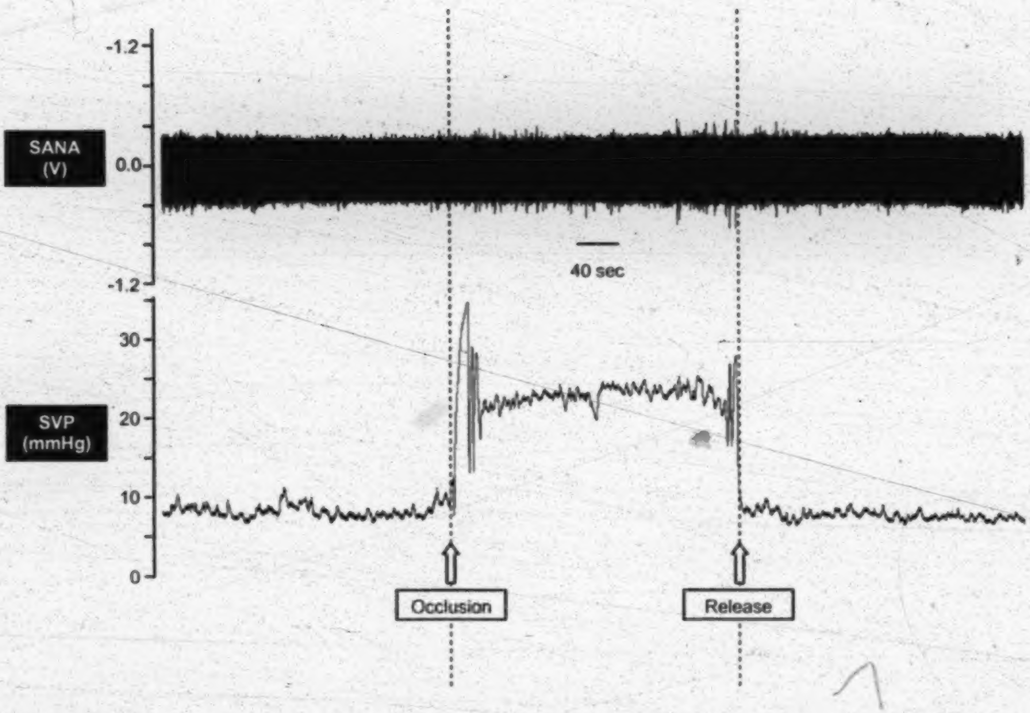


Figure 2-5. Representative raw data trace illustrating the changes in splenic afferent nerve activity (SANA, measured in volts) and splenic venous pressure (SVP, measured in mmHg) during splenic vein occlusion (SVO). After a 5 min baseline recording, the balloon occluder was inflated ("Occlusion") to partially occlude the splenic vein and attain a SVP of 20-24 mmHg. This pressure was maintained for 5 min, after which the balloon was deflated ("Release").

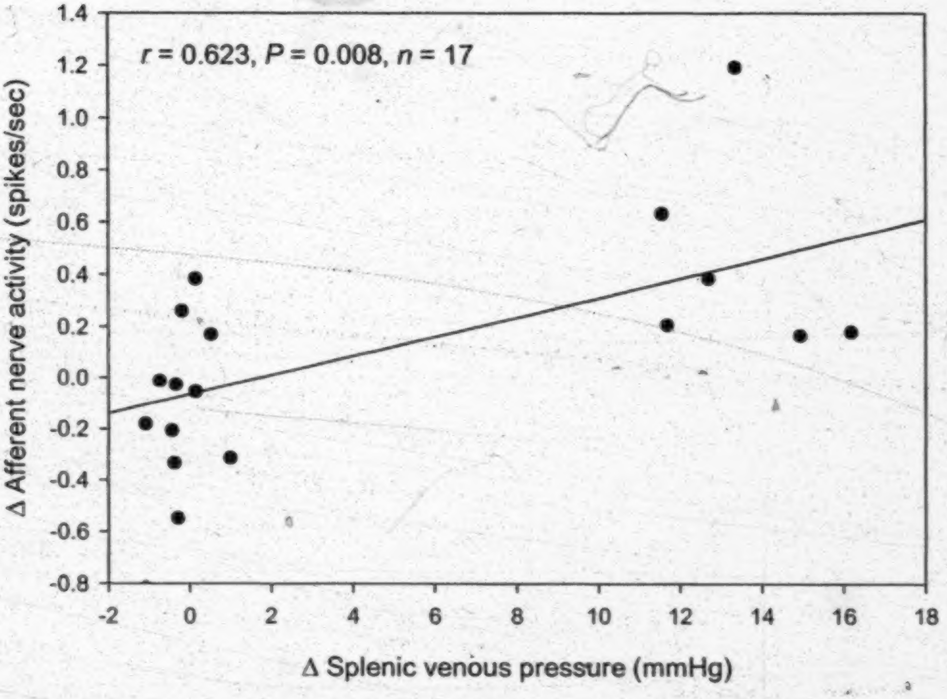


Figure 2-6. Linear regression analysis of the change in splenic afferent nerve activity (measured in spikes/sec) in response to the change in splenic venous pressure (measured in mmHg) observed during either splenic vein or splenic artery occlusion. Data points represent individual experiments in which either the splenic vein or the splenic artery was partially occluded, producing equivalent reductions in splenic arterial and venous blood flows.

BIBLIOGRAPHY

1. Andrews, W. H. and J. F. Palmer. Afferent nervous discharge from the canine liver. *Q J Exp Physiol Cogn Med Sci* 52: 269-76, 1967.
2. Armstrong, W. E., C. D. Sladek, and J. R. Sladek Jr. Characterization of noradrenergic control of vasopressin release by the organ-cultured rat hypothalamo-neurohypophyseal system. *Endocrinology* 111: 273-9, 1982.
3. Arroyo, V. and P. Gines. Mechanism of sodium retention and ascites formation in cirrhosis. *J Hepatol* 17: S24-8, 1993.
4. Baron, R. and W. Janig. Sympathetic and afferent neurons projecting in the splenic nerve of the cat. *Neurosci Lett* 94: 109-13, 1988.
5. Brookes, Z. L. and S. Kaufman. Effects of atrial natriuretic peptide on the extrasplenic microvasculature and lymphatics in the rat in vivo. *J Physiol* 565: 269-77, 2005.
6. Calaresu, F. R., J. C. Tobey, S. R. Heidemann, and L. C. Weaver. Splenic and renal sympathetic responses to stimulation of splenic receptors in cats. *Am J Physiol* 247: R856-65, 1984.
7. Chen, A. and S. Kaufman. Splenic blood flow and fluid efflux from the intravascular space in the rat. *J Physiol* 490 (Pt 2): 493-9, 1996.
8. Deng, Y., L. Copeman, and S. Kaufman. A novel natriuretic, hypotensive factor derived from the spleen. *Life Sci* 57: 1647-53, 1995.
9. Deng, Y. and S. Kaufman. Splenorenal reflex regulation of arterial pressure. *Hypertension* 38: 348-52, 2001.
10. DiBona, G. F. and L. L. Sawin. Hepatorenal baroreflex in cirrhotic rats. *Am J Physiol*

269: G29-33, 1995.

11. Elfvin, L. G., H. Aldskogius, and J. Johansson. Splenic primary sensory afferents in the guinea pig demonstrated with anterogradely transported wheat-germ agglutinin conjugated to horseradish peroxidase. *Cell Tissue Res* 269: 229-34, 1992.

12. Ermirio, R., P. Ruggeri, C. Molinari, and L. C. Weaver. Somatic and visceral inputs to neurons of the rostral ventrolateral medulla. *Am J Physiol* 265: R35-40, 1993.

13. Gupta, T. K., L. Chen, and R. J. Groszmann. Pathophysiology of portal hypertension. *Baillieres Clin Gastroenterol* 11: 203-19, 1997.

14. Hamza, S. M. and S. Kaufman. Splenorenal reflex modulates renal blood flow in the rat. *J Physiol* 558: 277-82, 2004.

15. Henderson, J. M., D. S. Barnes, and M. A. Geisinger. Portal Hypertension. *Curr Probl Surg* 35: 379-452, 1998.

16. Herman, N. L., D. R. Kostreva, and J. P. Kampine. Splenic afferents and some of their reflex responses. *Am J Physiol* 242: R247-54, 1982.

17. Jalan, R., E. H. Forrest, D. N. Redhead, J. F. Dillon, and P. C. Hayes. Reduction in renal blood flow following acute increase in the portal pressure: evidence for the existence of a hepatorenal reflex in man? *Gut* 40: 664-70, 199

18. Kaufman, S. and Y. Deng. Control of systemic blood pressure: influence of splenic afferent nerves and nitric oxide (NO). *The FASEB Journal* 13: A458, 1999.

19. Kaufman, S. and J. Levasseur. Effect of portal hypertension on splenic blood flow, intrasplenic extravasation and systemic blood pressure. *Am J Physiol Regul Integr Comp Physiol* 284: R1580-5, 2003.

20. Keenan, K. P., P. Laroque, and R. Dixit. Need for dietary control by caloric restriction

in rodent toxicology and carcinogenicity studies. *J Toxicol Environ Health B Crit Rev* 1: 135-48, 1998.

21. Kopp, U. C., L. A. Olson, and G. F. DiBona. Renorenal reflex responses to mechano- and chemoreceptor stimulation in the dog and rat. *Am J Physiol* 246: 67-77, 1984.

22. Kostreva, D. R., A. Castaner, and J. P. Kampine. Reflex effects of hepatic baroreceptors on renal and cardiac sympathetic nerve activity. *Am J Physiol* 238: R390-4, 1980.

23. Ludbrook, J. and W. F. Graham. The role of cardiac receptor and arterial baroreceptor reflexes in control of the circulation during acute change of blood volume in the conscious rabbit. *Circ Res* 54: 424-35, 1984.

24. Meckler, R. L. and L. C. Weaver. Characteristics of ongoing and reflex discharge of single splenic and renal sympathetic postganglionic fibres in cats. *J Physiol* 396: 139-53, 1988.

25. Moss, N. G. Electrophysiological characteristics of renal sensory receptors and afferent renal nerves. *Miner Electrolyte Metab* 15: 59-65, 1989.

26. Moursi, M. M. and L. G. D'Alecy. Depressor response during experimental portal hypertension in dog. *Am J Physiol* 251: H1188-94, 1986.

27. Nance, D. M. and J. Burns. Innervation of the spleen in the rat: Evidence for absence of afferent innervation. *Brain Behav Immun* 3: 281-90, 1989.

28. Niijima, A. Afferent discharges from venous pressoreceptors in liver. *Am J Physiol* 232: C76-81, 1977.

CHAPTER 3. SPLENORENAL REFLEX MODULATION OF CENTRAL CARDIOVASCULAR REGULATORY PATHWAYS

A version of this chapter has been submitted for publication.

Moncrief, Hamza & Kaufman 2006. Am J Physiol Regul Integr Comp Physiol.

3.1. INTRODUCTION

There is increasing evidence to suggest that the spleen is intimately involved in maintaining cardiovascular homeostasis. The spleen directly regulates plasma volume as a gateway for extravasation of protein-rich fluid from the intravascular space to the systemic lymphatics (14,37,39). There are several physiological and pathophysiological factors that may initiate and/or mediate this process, such as volume expansion, atrial natriuretic factor, nitric oxide, and endotoxemia (2,3,37,38). However, not only does the spleen directly control plasma volume, but it also provides tonic input to the maintenance of systemic blood pressure (23). Thus, it was found that inhibition of intrasplenic nitric oxide (NO) biosynthesis caused an increase in mean arterial pressure (MAP), whereas significant hypotension was observed secondary to the effects of portal (and splenic venous) hypertension (30). Our recent finding that splenic baroreceptors control splenic afferent nerve activity (50) suggests a mechanism by which the spleen may act as a monitor of the splanchnic circulation, a vascular bed which is crucial to the maintenance of cardiovascular homeostasis (29,45).

Increased activity of splenic baroreceptors reflexly increases renal sympathetic nerve activity (RSNA) through a spinal reflex pathway known as the splenorenal reflex (33,47,50). Activation of the splenorenal reflex by partial occlusion of the splenic vein invariably activates both splenic and renal nerves through: direct excitation of splenic afferent fiber endings in the wall of the splenic vein (33), and indirect activation of renal afferents (1). This latter response is initiated by the increase in RSNA which, through its effects on renal vascular tone, reflexly increases renal afferent nerve activity (41). Renal chemoreceptors are responsive to renal ischemia, such as that caused by a reduction in renal blood flow, and are known to project centrally (1,7,52,59,60,71). It is evident then, that any systemic cardiovascular effects generated by the splenorenal reflex could be mediated by central pathways activated by the spleen and/or the kidney. Furthermore, these projections may contribute to the central cardiovascular dysregulation observed in portal hypertension (70).

The present study was designed to investigate these possibilities by examining whether stimulation of splenic baroreceptors activates known cardiovascular regulatory centres in the brain and, furthermore, if central neural activation under these circumstances is affected by prior renal and/or splenic denervation. It was our objective to determine the relative contribution of the spleen and kidney to central signaling during splenic vein occlusion (SVO). In separate groups of animals, we examined the effect of SVO in: intact rats; bilaterally renal-denervated rats (to remove renal afferent input, thereby isolating the effect of splenic afferent activity); and renal + splenic-denervated rats (to determine if there was any non-neural contribution).

In this study, we have focused on the main brainstem and hypothalamic nuclei subserving autonomic and neuroendocrine regulation of the cardiovascular system, including the nucleus of the tractus solitarius (NTS), ventrolateral medulla (VLM), paraventricular nucleus (PVN) and supraoptic nucleus (SON) (Fig. 3-1). The NTS is the primary relay station through which cardiovascular afferent inputs (ex. baroreceptor, chemoreceptor and volume receptor reflexes) are received in the brainstem and subsequently transmitted to higher brain centres such as the hypothalamus, either directly or indirectly via the VLM (18,72). The VLM directly regulates sympathetic outflow to the periphery, specifically through an area known as the rostral ventrolateral medulla (RVLM) (18,72). The RVLM has direct spinal projections to preganglionic sympathetic neurons and activation of this region is known to elicit increases in systemic blood pressure (12,13,17,72). The VLM and NTS are reciprocally interconnected, facilitating their role in regulating autonomic signaling between the CNS and periphery (15,18). Communication between these brainstem centers involves an excitatory pathway from the NTS to the caudal ventrolateral medulla (CVLM), which in turn, inhibits presympathetic neurons in the RVLM (15,18). Likewise, activation of neurons within the CVLM has been shown to elicit a depressive cardiovascular effect, with a decrease in systemic blood pressure, heart rate and sympathetic activity (15,18). In addition to their reciprocal communication, the NTS and VLM are integral players in hypothalamic autonomic regulation through their projections to the PVN, and are known to respond vigorously to cardiovascular manipulations such as hemorrhage or infusions of vasoactive agents (18,34,42,72).

The PVN integrates a variety of inputs from autonomic centers in the spinal cord, brainstem, and forebrain (74) (Fig. 3-1). Also projecting to the PVN are the circumventricular organs, including the subfornical organ of the forebrain (SFO) and organum vasculosum of the lamina terminalis (OVLT) (Fig. 3-1); these structures lack a blood-brain barrier, and are thus directly affected by circulating messengers in the bloodstream such as angiotensin II (ATII) (48). Correspondingly, these regions are known to have a high density of ATII receptor binding sites (58). The PVN is divided into magnocellular (MC) and parvocellular (PC) components, distinguishable on the basis of anatomical location and cellular content (73).

Neurons from the MC division of the PVN mainly project to the posterior pituitary and produce either oxytocin (OXY) or vasopressin (AVP) (19,73). With a similar neurosecretory role involved in the maintenance of body fluid homeostasis, the SON receives a variety of input from ascending brainstem pathways activated by baroreceptors and volume receptors, and from direct projections from the circumventricular organs (54,67,68). This nucleus consists of large OXY- and AVP-producing cells that project to the neural lobe of the pituitary gland (54). Stimulation of renal afferents has been shown to induce activation of the SON leading to increased AVP secretion (16,20,40).

The PC division of the PVN consists mainly of neurons projecting to extrahypophysial sites and can be topographically subdivided (62,73). Common throughout each division is the presence of some posterior pituitary-projecting cells containing AVP or OXY (73,74,80). The majority of neurons located medially within the PC PVN project to the median eminence where they may release a number of hormonal factors important in cardiovascular regulation. For example, secreted corticotrophin releasing factor (CRF) is important in the subsequent release of adrenocorticotropic hormone (ACTH) and its cardiovascular effects (62). Descending projections to autonomic cell groups in the brainstem and spinal cord from lateral and medial portions of the PC PVN have also been described. The majority of neurons projecting to the brainstem arise from the anterior region of the PC PVN, while projections to preganglionic autonomic neurons in the intermediolateral cell column of the spinal cord originate in the dorsal aspect (54,66,67,73).

Immunohistochemical detection of Fos, the protein product of the immediate-early gene *c-fos,* is an established method of identifying neurons that have been activated by physiological and pathophysiological stimuli (24,51,58,62,65). The objective of this study was to examine the pattern of central neural activation in response to stimulation of splenic baroreceptors via partial occlusion of the splenic vein. To determine the peripheral origin of central activation we studied Fos activation in pairs of control vs. splenic vein-occluded rats in intact, renal-denervated and renal + splenic-denervated animal groups.

3.2. METHODS

All experimental procedures carried out in this study were examined and approved by the local Animal Welfare Committee according to the guidelines set by the Canada Council on Animal Care.

3.2.1. Animals and housing

Male Long Evans rats (300-500 g) were purchased from Charles River Canada (St. Foy, QC, Canada) and housed in the University Animal Facility in a temperature-controlled environment, exposed to light on a 12:12-h light-dark cycle, and maintained on a 0.3% sodium diet and water ad libitum. The study was comprised of three experimental groups which were further divided into pairs of control and experimental subsets: *1) Intact*: non-occluded controls ($n = 6$) and splenic vein-occluded rats ($n = 5$); *2) Renal-denervated*: non-occluded controls ($n = 5$) and splenic vein-occluded rats ($n = 6$); and *3) Renal + splenic-denervated*: non-occluded controls ($n = 6$) and splenic vein-occluded rats ($n = 5$).

3.2.2. Drugs

All surgical procedures were carried out aseptically under sodium pentobarbital anesthesia (62 mg/kg body wt, ip; MTC Pharmaceuticals, Cambridge, ON, Canada). Atropine sulphate (Sabex, Boucherville, QC, Canada) was injected intramuscularly (0.10 mg/kg body wt) to minimize bodily secretions during surgery. The proteinase inhibitor, aprotinin (Trasylol, 0.25 ml, ip), was administered locally during surgery to minimize damage arising from pancreatic tissue manipulation. Temperature was maintained using Delta-phase heating pads (Braintree Scientific, Braintree, MA). Following surgery, all animals were given an analgesic, buprenorphine hydrochloride (Buprenex injectable, 0.02 mg/kg body weight, im; Reckitt and Colman Pharmaceutical, Richmond, VA), for post-operative pain management.

3.3.3. Surgery and Chronic Instrumentation

3.3.3.1. Implantation of PA-C10 pressure transmitter

A midline incision was made to expose the spleen and its vasculature. A PA-C10 telemetric pressure transducer (1.1cc, 0.4 mm ID; Data Sciences International, Arden Hills, MN) was inserted occlusively into the gastric vein and its tip advanced to the junction with the splenic vein for continuous measurement of splenic venous pressure (SVP) in the conscious rat. During the *in vivo* experimental protocol, SVP was transmitted to a signal receiver, which was connected to a PC for online data acquisition using WINDAQ/Pro software (DATAQ Instruments, Akron, OH).

3.3.3.2. Implantation of vascular balloon occluder for splenic vein occlusion

A vascular balloon occluder (VO-1.5N, No. 18080-01; Fine Science Tools, North Vancouver, BC, Canada) was implanted and secured around the splenic vein between the gastric and hepatic portal vein. A rectangular piece of fine mesh (1 x 2 cm) with smoothed edges was secured to the actuating tube at the base of the occluder cuff to provide a flat base preventing rotational movement of the occluder. The actuating tube was passed through the abdominal muscle and exteriorized at the nape of the neck via 16G hypodermic stainless steel tubing (Small Parts, Inc., Miami Lake, FL), providing a means for inflation and controlled partial occlusion of the splenic vein in the conscious animal.

3.3.3.3. Renal and/or splenic denervation

In the RD group of animals, bilateral renal denervation was carried out to isolate nervous activity from the spleen and remove any central effects triggered by renal afferent nerves. The renal nerves were carefully stripped away from the renal vessels and sectioned. In the RSD group, the spleen was also denervated to act as a negative control (ie. unmask any background central effects on *c-fos* originating from elsewhere than splenic or renal afferents). The splenic nerve bundle was exposed between the splenic artery and vein, isolated, and sectioned. After cutting away all visible nerve fibers in either case, a 10% phenol solution was painted on the area to ensure complete destruction of the renal and/or splenic nerves. Caution was taken to avoid damaging the celiac ganglion.

3.3.3.4. Non-occlusive cannulation of abdominal aorta

Micro-Renathane tubing (MRE-033; 0.36 mm ID, 0.84 mm OD; Braintree Scientific, Braintree, MA) was inserted non-occlusively into the abdominal aorta and secured to the psoas muscle. The cannula was exteriorized at the nape of the neck via 27G stainless steel tubing (Small Parts, Inc., Miami Lake, FL) and filled with heparinized saline (10,000 units/l) to maintain patency. During the *in vivo* experiment, the exteriorized aortic catheter was attached to a Gould Statham pressure transducer (Gould Electronics, Recording Systems Division, Cleveland, OH) for online monitoring of mean arterial pressure using WINDAQ/Pro software (DATAQ Instruments, Akron, OH).

3.3.4. Experimental Protocol

Animals recovered in their own cage following surgery and on the fourth day were relocated to a metabolic cage for acclimatization to experimental conditions. On the fifth day, the gastric transmitter was turned on and the exteriorized aortic catheter (PE-20; 0.38 mm ID, 0.43 mm OD; Becton Dickinson, Sparks, MD) attached to a pressure transducer for online recording of splenic venous pressure (SVP) and mean arterial pressure (MAP), respectively. The exteriorized balloon occluder catheter (Tygon EW-06418-04, 1.02 mm ID, 1.88 mm OD; Cole-Palmer, Anjou, QC, Canada) was attached to a 10cc syringe filled with isotonic saline for inflation purposes. Conscious animals were allowed to stabilize for ~30 minutes, followed by a 30 minute baseline recording. In splenic vein-occluded animals, the balloon occluder was then inflated and clamped to increase and maintain SVP at 20-30 mmHg for 1 hour. This value was chosen according to a previously established protocol for partial SVO, mimicking the effects of portal hypertension (30). In slight contrast to our previous experiments carried out in anesthetized animals, we chose a slightly elevated pressure to simulate portal hypertension in this conscious study. Control (non-occluded) animals received the same treatment with exception of balloon inflation. After 1 hour, rats received an anesthetic overdose of pentobarbital sodium (50 mg/kg body wt, ip, MTC Pharmaceuticals, Cambridge, ON, Canada) and were prepared for fixation.

3.3.5. Tissue processing

Animals were perfused transcardially with ~200 ml ice-cold 0.9% physiological saline to clear the system of blood, followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.2) to fix the tissues. Brains were removed and post-fixed in a 1:1 solution of 30% sucrose and 4% paraformaldehyde for 1 hour at 4°C. Brains were then transferred to 20% sucrose for cryoprotection overnight at 4°C. The following day, serial transverse sections (40 μm) of hypothalamus and brainstem were cut in a cryostat and placed free-floating in phosphate-buffered saline (PBS, pH 7.2) for subsequent immunohistochemical processing.

3.3.6. Fos immunohistochemistry

Fos staining was carried out using the Vectastain Elite ABC Kit (Rabbit IgG) (PK-6101, Vector Laboratories, Burlingame, CA). Floating sections were incubated in PBS-diluted Vectastain blocking serum (1.5% normal goat serum) for 30 minutes at room temperature. The tissue was rinsed and transferred to a 1:200 solution of primary antibody, anti *c-fos* [Ab-2], polyclonal rabbit IgG (PC05, Calbiochem/EMD Biosciences, San Diego, CA), in 0.3% Triton X-100/PBS. Due to enhanced sensitivity of the Vectastain Elite ABC Kit permitting shortened primary antibody incubation times, sections were incubated in primary antibody for 1 hour at room temperature with agitation. Punctuated with 10 minute PBS rinses, tissues were incubated sequentially for 1 hour at room temperature in PBS-diluted 1:200 Vectastain biotinylated secondary antibody solution (Biotinylated Goat Anti-Rabbit IgG) combined with Vectastain normal blocking serum, followed by PBS-diluted 1:100 Vectastain Elite ABC Reagent (avidin-biotin peroxidase complex). Sections were then washed in PBS and incubated in diaminobenzidine peroxidase for 10 minutes at room temperature to visualize Fos labeling as a brown reaction product. Sections were rinsed in PBS and mounted on glass microscope slides, air-dried overnight, and coverslipped.

3.3.7. Quantification and analysis

Neurons labeled for Fos were examined with light microscopy by counting labeled cell nuclei in the NTS, VLM, PVN and SON manually (representative photomicrographs, Fig. 3-2). All slides were analyzed together to decrease variability leading to counting error. Relevant hypothalamic and brainstem nuclei were identified in accordance with the atlas of Paxinos and Watson (57). Every other section was analyzed and counts from both hemispheres were combined for each section per animal. Data were then averaged for control and experimental groups to obtain the number of neurons per section positive for Fos. To aid in analysis, brainstem regions were objectively divided into three levels: rostral (-11.60 mm bregma), intermediate (-13.30 bregma) and caudal (-14.60 bregma). Since the intermediate-caudal regions of the NTS have previously been identified as critical for cardiovascular regulation (63), only sections between -14.30 mm to -12.80 mm bregma were analyzed, for an average of 21 ± 2 sections per animal. The VLM was analyzed throughout its rostral-caudal distribution, from -11.60 mm to -14.60 mm bregma, for an average of 30 ± 2 sections per animal. The PVN was analyzed at approximately -1.80 mm bregma, corresponding to an intermediate level (ie. mid-PVN) which is divisible into two subregions consisting of MC and PC neurons. On average, 14 ± 1 sections were analyzed for Fos labeling in the PVN of each animal. Labeled neurons of the SON were counted throughout the rostral-caudal distribution from -0.80 mm to -1.80 mm bregma, corresponding to 30 ± 1 sections per animal.

3.3.8. Statistical Analysis

Results are presented as means ± SE. *In vivo* blood pressure data were analyzed for multiple comparisons between groups using 2-way repeated-measures analysis of variance (ANOVA). Differences in numbers of labeled neurons between groups and variations in rostral-caudal distribution of labeled neurons were analyzed by two-way ANOVA. The Bonferroni t-test was used for post hoc analysis of multiple comparisons versus controls. For all tests, statistical significance was accepted at $P < 0.05$.

3.3. RESULTS

3.3.1. Hemodynamic measurements

Baseline blood pressure data represent the average of a 5 minute period immediately preceding the occlusion stimulus. Occluded values represent the average of a 5 minute period ~30 minutes before the end of the occlusion stimulus, corresponding to a time point ~45 minutes before tissue fixation.

Comparisons of baseline values (prior to vessel occlusion) revealed no significant differences between any of the experimental groups with respect to MAP or SVP (Table 3-1). In addition, statistical analysis of control (non-occluded) groups revealed no significant changes in MAP or SVP from baseline values over time. For these reasons, results for blood pressure responses are presented as a change from baseline for all experimental groups.

3.3.1.1. Splenic venous pressure

Inflation of the balloon occluder around the splenic vein significantly increased SVP in all three experimental groups compared to baseline values (Fig. 3-3). Furthermore, there were no significant differences among SVO groups with respect to baseline or occlusion values of SVP (ie. equivalent stimulus for splenic baroreceptor activation). No significant changes were observed for the three (non-occluded) control groups with respect to SVP (Fig. 3-3).

3.3.1.2. Mean arterial pressure

Partial occlusion of the splenic vein in the conscious rat significantly increased MAP from baseline in the intact animals (115.7 ± 1.7 mmHg to 124.4 ± 2.6 mmHg; $n = 5$; $P = 0.025$) (Fig. 3-4). However, this change from baseline was not significant relative to the intact non-occluded controls. Within the groups, there were no significant changes in MAP from baseline for renal-denervated (RD) or renal + splenic-denervated (RSD) animals, or for any of the (non-occluded) control groups, ie. the increase in MAP observed with SVO in the intact animals was abolished with renal denervation alone and in the renal + splenic-denervated animals (Fig. 3-4).

3.3.2. Effect of denervations on numbers of Fos-positive neurons in controls

3.3.2.1. NTS

Analysis of rostral-caudal distribution of labeled neurons in the NTS revealed no demonstrable variation between levels (data not shown). Data are therefore presented as the number of labeled nuclei per section for the intermediate-caudal regions. As shown in Fig. 3-5 (open bars), Fos staining was prevalent in the intact control animals. There was no difference in renal-denervated controls as compared to the intact group. There was, however, a significantly reduced number of neurons labeled for Fos in renal + splenic-denervated controls compared to both intact and renal-denervated groups (RSD, $n = 5$; $P < 0.001$ vs. intact and RD controls).

3.3.2.2. VLM

There was no significant rostral-caudal variation of labeled neurons in the VLM (data not shown). Data are therefore presented as the number of labeled nuclei per section for the entire region. There was abundant Fos staining in the intact controls (Fig. 3-6). Renal denervation did not significantly alter the number of neurons labeled for Fos as compared to intact controls (open bars); however, renal + splenic denervation slightly increased the number of labeled neurons (RSD, $n = 6$; $P = 0.018$ vs. intact controls). There was no significant difference between renal-denervated and renal + splenic-denervated controls.

3.3.2.3. PVN

There were no differences between control groups (intact, RD, RSD) with regard to numbers of Fos-labeled neurons in the PC region of the PVN (Fig. 3-7*A*, open bars). Similarly, denervation had no effect on Fos labeling in MC neurons of the PVN (Fig. 3-7*B*, open bars).

3.3.2.4. SON

Neurons labeled for Fos were evident in the SON of intact control animals (Fig. 3-8, open bars). In comparison to intact controls, renal denervation tended to increase the number of labeled neurons, but this failed to reach significance (RD, $n = 6$; $P = 0.075$ vs. intact controls). Renal + splenic denervation slightly increased the number of Fos-positive neurons as compared to intact controls (RSD, $n = 5$; $P = 0.005$ vs. intact controls), but this was not significantly different from renal denervation alone.

3.3.3. Effect of splenic vein occlusion on numbers of Fos-positive neurons

3.3.3.1. NTS

Partial occlusion of the splenic vein did not significantly alter the number of Fos-positive neurons in the NTS of intact animals (Fig. 3-5, solid bars). In contrast, a significant reduction in the number of labeled neurons was observed with SVO in the renal-denervated group (RD, $n = 6$; $P < 0.001$ vs. RD controls). When the spleen was also denervated, SVO caused no change in the number of Fos-labeled neurons compared to RSD controls.

3.3.3.2. VLM

SVO did not significantly alter the number of labeled neurons in the VLM in intact animals (Fig. 3-6, solid bars). In contrast, both renal- and renal + splenic-denervated animals showed significant reductions in Fos compared with their non-occluded counterparts (RD, $n = 6$; $P < 0.001$ and RSD, $n = 5$; $P = 0.003$). There was, however, no difference between renal denervation alone and renal + splenic denervation.

3.3.3.3. PVN

There was a large increase in the number of Fos-positive neurons in the PC region of the PVN in intact animals with SVO (intact, $n = 5$; $P < 0.001$ vs. intact controls) (Fig. 3-7*A*, solid bars). Renal denervation abolished this response. Denervation of the spleen had no added effect on the number of labeled neurons after SVO. Partial SVO caused a significant increase in the number of MC neurons of the PVN labeled for Fos in intact animals (intact, $n = 5$; $P = 0.012$ vs. intact controls) (Fig. 3-7*B*). Similar stimulation was observed with SVO in the renal-denervated animals (RD, $n = 6$; $P < 0.001$ vs. RD controls). In contrast, denervation of the spleen abolished this effect, with no change in numbers of labeled neurons observed with SVO. A representative photomicrograph (Fig. 3-9) illustrates the observed increase in Fos labeling in both regions of the PVN in intact SVO animals vs. intact controls.

3.3.3.4. SON

SVO caused a significant increase in the number of Fos-positive neurons in the SON of intact animals (intact, $n = 5$; $P < 0.001$ vs. intact controls) (Fig. 3-8, solid bars). Renal denervation abolished this response. With added splenic denervation, there was a significant reduction in the number of labeled neurons in the SON observed with SVO (RSD, $n = 5$; $P =$

0.004 vs. RSD controls). Fig. 3-9 demonstrates an increased number of Fos-positive neurons in the SON of intact SVO animals vs. intact controls.

3.4. DISCUSSION

We have previously demonstrated that partial occlusion of the splenic vein causes an immediate increase in splenic venous pressure which is accompanied by an increase in splenic afferent nerve activity (30,50). It is well known that enhanced splenic afferent nerve activity causes a reflex increase in renal sympathetic outflow through a spinal pathway leading to a reduction in renal blood flow (8,30,33). Since studies had shown that portal hypertension (caused by portal vein stenosis) alters central *c-fos* expression (70), we wished to investigate the overall role of the splenorenal reflex and moreover, the individual contributions of the spleen and kidney in this central dysregulation. We found that partial occlusion of the splenic vein in the conscious rat *does* affect central neuronal activation, the effect of which depends on the integrity of renal and splenic innervation. The majority of direct effects can be attributed to renal afferent input, while splenic afferent input may be best explained by indirect effects of a reflex change in cardiac preload. However, evidence for a direct central pathway from the spleen remains inconclusive.

In order to tease apart the effect of splenic vein occlusion (SVO) on central activation, it was necessary to first assess the effect of chronic renal and/or splenic denervation on basal *c-fos* expression, which required a review of renal afferent input to the brain. It is established that information from the kidneys is sent to the brain both by an afferent neural pathway consisting of spinal sympathetic afferent nerves and by a humoral pathway activated by renin release and subsequent formation of angiotensin II (ATII) (26). Primary renal afferents are located in the dorsal root ganglia of the caudal thoracic and rostral lumbar spinal cord

(15,28,61) and second-order spinal afferents are found in the dorsal horn at these same levels (25,27,52). Renal afferent nerves project directly to the NTS (69) and to several regions of the anterior hypothalamus, including the PVN and SON (71). Sensory information from the kidney selectively excites vasopressin (AVP)-secreting neurons, as evidenced by an increase in plasma AVP following electrical stimulation of renal afferent nerves (71). The central effects of the humoral pathway are mediated by ATII, which is known to induce Fos in the SON, PVN, OVLT and SFO (32,43,46).

Although there is a wealth of information regarding the cardiovascular effects of chronic bilateral renal denervation, relatively few studies have focused on the changes to central neural activation. However, a two-part study by Fitch *et al.* (2000) examining the effect of renal denervation and angiotensin blockade on activation of renal afferents by furosemide, serves as an adequate comparison to our studies. Furosemide, a known stimulus for renal afferent nerves, induced *c-fos* expression in the SFO, OVLT, SON, magnocellular (MC) PVN and NTS of control rats (26). Bilateral renal denervation abolished this effect *only* in the NTS, suggesting that activation in the NTS is due to the renal sympathetic afferent pathway, while activation of the forebrain regions depends more so on the humoral pathway activated by ATII (26). In relation to renal-induced central effects, the PVN and SON of the hypothalamus are known to be affected by at least three neural pathways: *1)* direct connection from the SFO/OVLT mediated by ATII (44,64,77); *2)* baroreceptor-mediated pathway via activation of low-pressure or volume receptors, relayed through the NTS and noradrenergic A1 cell group of the VLM (4-6); and *3)* direct projection of renal afferents (9-11,20,21).

Renal denervation in our control groups did not significantly alter basal brainstem levels of Fos. However, when both the kidneys and the spleen were denervated, we saw significantly reduced neuronal activation in the NTS as compared to intact controls. This was a surprising find, suggesting that the spleen's input to the NTS has a greater influence than the kidneys. However, renal afferents are known to have an array of projection targets in the CNS, thereby distributing renal input throughout the brain (71). Splenic input may be more localized to the NTS, and its removal may therefore directly reduce basal neuronal activity, or conversely, may unmask an inhibitory influence to the NTS.

Chronic renal and/or splenic denervation is known to cause chronic systemic hypotension (23,35). It is assumed that interruption of the renal nerves produces a long-term decrease in MAP as a combined result of losing renal afferent and efferent innervation (35). Renal afferents modulate AVP release and systemic sympathetic activity, while renal efferents maintain renal vasomotor tone and mediate secretion of renin (35). We saw a graded increase in basal Fos expression in the SON and VLM with renal denervation and renal + splenic denervation as compared to intact controls. SON neurons are AVP and/or OXY-secreting (75), while VLM controls sympathetic outflow (17); enhanced activity in these regions may suggest a compensatory mechanism by which MAP was maintained despite renal and/or splenic denervation. A similar trend was observed in both PC and MC regions of the PVN. Although we did not observe a fall in resting MAP in the denervated animals, blood pressure may have been maintained by the observed increase in central activation.

We found that with renal and splenic nerves intact, partial occlusion of the splenic vein produced a small increase in MAP. This effect appears to be mediated mainly by the renal nerves, as renal denervation alone abolished the response. It is likely that this slight elevation of systemic blood pressure is a result of the splenorenal reflex: increased sympathetic outflow to the kidneys enhances renin secretion, leading to elevated circulating levels of ATII (23). The increased circulating ATII would directly raise MAP through its peripheral actions on the vasculature. In addition, there would be ATII-induced central activation of forebrain, but not hindbrain neural centres (18). This is consistent with our finding that there was an SVO-induced increase in Fos expression in the PVN and SON, but not in the NTS or VLM. Furthermore, the fact that we did not observe enhanced Fos expression in the NTS or VLM with SVO render it unlikely that compensatory baroreflex mechanisms were directly activated by this small increase in MAP.

Not only was Fos expression in the NTS unaffected by a small increase in MAP, but the increase in splenic afferent nerve activity also failed to induce an increase in NTS Fos. These data suggest a lack of direct input from the splenic afferent nerves to the NTS, which is consistent with evidence that visceral afferents reach the brainstem primarily via the vagus (Xth cranial) nerve and that few, if any, vagal afferents innervate the spleen (53). The NTS is, however, the site of termination of visceral afferent fibers of the IX and Xth cranial nerves, which carry baroreceptor information from the carotid sinus and aortic depressor nerves, as well as from the cardiopulmonary afferents and atrial volume receptors (22,56,63). It therefore appears that NTS activity is predominantly influenced by cardiac receptor and baroreceptor afferents, with negligible input from only a small number of splenic afferent fibers.

Interestingly, SVO in the renal-denervated animals significantly *depressed* Fos expression in the NTS, and this depression was abolished with additional splenic denervation. These results suggest that input from the spleen has an inhibitory effect on neuronal activity in the NTS, which is masked in the intact group by excitatory renal afferent input. We observed similar inhibition of neuronal activity in the VLM with renal denervation, but in contrast to the NTS, this effect persisted after additional splenic denervation. Due to the complexity of the multi-synaptic pathways through which the VLM is involved, the source of this depression remains to be determined. It is also difficult to conclude whether the inhibitory effect on the NTS is a direct result of splenic afferent projections to the NTS (unlikely), or indirect through an unidentified peripheral mechanism. A candidate site for peripheral inhibition is the mesenteric vasculature, evidenced by ongoing discharge of mesenteric nerves after decentralization (47) or blockade of rostral VLM sympathetic outflow (31). Mesenteric nerve activity (and splanchnic vascular resistance) is thus believed to be controlled, at least in part, by peripheral non-adrenergic, non-cholinergic (NANC) sources (49).

Neurotransmitters released by NANC nerves, including calcitonin-gene-related peptide (CGRP) and nitric oxide (NO), are known to induce vasodilation of the splanchnic vascular bed (49). Splanchnic compliance and blood volume mobilization are controlled mainly through sympathetic innervation (29); furthermore, vasodilatory responses to NANC nerve stimulation are demonstrated only after vessel preconstriction (49). These findings suggest that NANC-induced vasodilation is due to inhibition of sympathetic tone in the splanchnic vascular bed. We propose that this inhibitory effect may be mediated through a putative spleno-intestinal reflex, whereby an increase in splenic afferent nerve activity causes reflex *vasodilation* of the mesenteric vasculature, leading to splanchnic blood pooling and a subsequent reduction in cardiac preload (Fig. 3-10). This would reduce atrial volume receptor afferent nerve activity, which is known to project to the NTS, thereby reducing NTS activation (22,56).

Decreased splanchnic vascular resistance is a hallmark of portal hypertension and furthermore, is believed to be an initiating factor for development of a hyperdynamic circulatory state (36,49,76,78). It is of considerable interest that NANC-induced vasodilation of mesenteric arteries is exacerbated in portal hypertensive rats (36,49). Nitrergic nerves, containing neuronal nitric oxide synthase (nNOS), and CGRP-releasing nerves are stimulated in mesenteric arteries in portal hypertension and have been implicated in the profound splanchnic vasodilation typical of this condition (36,49). Our proposed mechanism of mesenteric vasodilation through a spleno-intestinal reflex may explain how an increase in splenic venous pressure secondary to increased portal venous pressure, may contribute to the pathogenesis of portal hypertension by exacerbating splanchnic pooling and central underfilling.

In contrast to the brainstem regions studied, SVO significantly enhanced Fos expression in the intact animals in all hypothalamic nuclei investigated. Again, it is unlikely that this hypothalamic activation was secondary to the small increase in blood pressure, as hypertension alone does not directly induce Fos expression in the hypothalamus (18). However, since ATII is known to act directly at the SFO and OVLT, indirectly activating pathways to the PVN and SON (55,81), we suggest that increased activation in these regions was a combined result of splenorenal reflex production of circulating ATII and direct activation by renal afferent innervation (71) (Fig. 3-10). The response of parvocellular (PC) PVN neurons to partial splenic vein occlusion appears entirely dependent on intact renal nerves, as the effect is abolished with renal denervation and no further effect is observed with splenic denervation. On the other hand, enhanced activity of magnocellular (MC) neurons persists after renal denervation, but is abolished when splenic neural input is removed. We suggest that splenorenal effects on PC PVN neurons originate in the kidney, whereas the effects on MC PVN neurons are likely more dependent upon direct input from splenic afferent nerves. This difference may reflect the functional differences of PC PVN vs. MC PVN neurons; PC PVN neurons exert their actions through ascending pathways to the anterior pituitary and descending pathways to the brainstem and spinal cord (74), whereas MC PVN neurons project to the posterior pituitary, causing release of vasopressin (AVP) and/or oxytocin (OXY) (74). We may further hypothesize that while PC PVN activation reflects renal vasoconstriction and blood pressure response, MC PVN activation mirrors splanchnic congestion and blood volume response.

Neurons of the SON are functionally similar to MC neurons of the PVN, projecting to the posterior pituitary where they release AVP and OXY (79). SVO also enhanced Fos expression in the SON of intact animals. Stimulation of renal afferents is known to activate SO nuclei and excitatory input would also be received from the SFO and OVLT in response to increased plasma ATII; both of these inputs would lead to increased AVP release from the SON (20). Since bilateral renal denervation abolished the augmented activity of the SON, we suggest that the majority of direct neural input in response to SVO arises from the kidney. In contrast, renal + splenic denervation had very little effect on neuronal activity in the SON in response to SVO, which suggests a lack of direct neural input from the spleen to this region. Given the functional similarities between MC neurons of the PVN and the SON, we expected a similar response to renal and/or splenic denervation and to SVO between the two regions. Both of these hypothalamic nuclei play an important role in cardiovascular homeostasis through regulation of blood volume. It is already known that direct neural input from the kidneys is received at the SON (20). We suggest that the spleen, as an accessory to the kidneys in blood volume regulation, contributes to volume homeostasis through a direct neural connection to the MC region of the PVN. Together, information from the spleen and kidneys is integrated in the hypothalamus and subsequently translated into hormonal control of blood volume through the posterior pituitary. When both renal and splenic input was removed, we observed a slight depression of neuronal activity in the SON. It is tempting to speculate that this depression may be the result of released humoral factors and/or altered hepatic hemodynamics and subsequent afferent nerve activity downstream from the splenic vein occlusion. Conversely, this slight depression may, in fact, be an artifact of increased basal *c-*

fos in the RSD group. It is, however, difficult to draw firm conclusions based on the results of the present study.

The objective of this study was to determine whether the splenorenal reflex influences central neural activation which then mediates systemic cardiovascular effects. The results indicate that the splenorenal reflex does exert its complex effects through activation and/or inhibition of central pathways affecting cardiovascular regulation (Summary, Fig. 3-10). However, the specific pathways by which these actions take place remains to be determined. Future studies are required to narrow down the possibilities for our observed results. Double-labeling for specific neuronal cell types would aid in identifying the specific pathways involved; retrograde tracing would provide a means of directly identifying the source of neuronal activation in the splenorenal reflex. In conclusion, we have shown that activation of splenic baroreceptors and the splenorenal reflex leads to a specific pattern of central neural activation, which depends upon a combination of direct and indirect input from renal and splenic afferents, as well as possible indirect effects through modulation of other neural or non-neural stimuli to the brain.

3.5. ACKNOWLEDGEMENTS

We thank Dr. T. Krukoff for her helpful comments about these experiments, in addition to offering the use of her cryostat for tissue processing. We also acknowledge technical assistance in immunohistochemical methods provided by Heather Edgell. This research was supported by grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Alberta, Northwest Territories, and Nunavut, Canada.

FIGURES

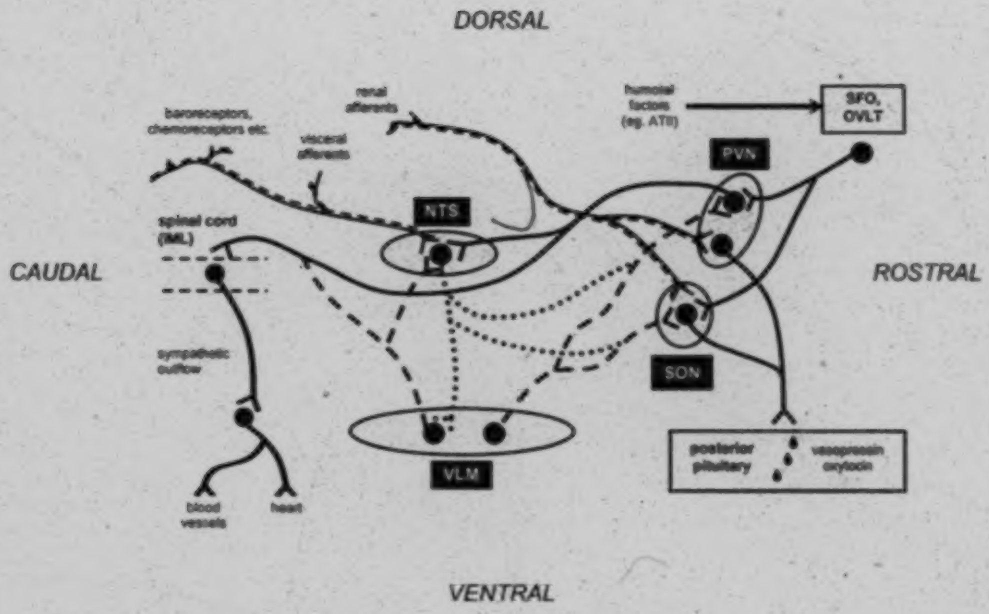

DORSAL
CAUDAL
ROSTRAL
VENTRAL
baroreceptors, chemoreceptors etc.
renal afferents
visceral afferents
humoral factors (eg. ATII)
SFO, OVLT
PVN
NTS
spinal cord (IML)
sympathetic outflow
SON
VLM
blood vessels
heart
posterior pituitary
vasopressin oxytocin

Figure 3-1. Schematic of a sagittal view of the brain illustrating the main nuclei and neural pathways involved in autonomic and neuroendocrine control of the cardiovascular system. Sensory input from cardiovascular and visceral receptors is received and relayed between the brainstem and hypothalamic cardiovascular regulatory centres, ultimately controlling sympathetic outflow to the periphery. Peripheral afferent nerves are shown as double solid/dashed lines; peripheral sympathetic efferent nerves as double solid lines. Neural output from the NTS is illustrated by dotted lines; VLM output by dashed lines; forebrain and hypothalamic output by solid lines. Abbreviations: IML, intermediolateral cell column; NTS, nucleus of the tractus solitarius; PVN, paraventricular nucleus; VLM, ventrolateral medulla; SON, supraoptic nucleus; SFO, subfornical organ; OVLT, organum vasculosum of the lamina terminalis. Note: Schematic is not drawn to scale and locations of nuclei are approximated for illustration purposes.

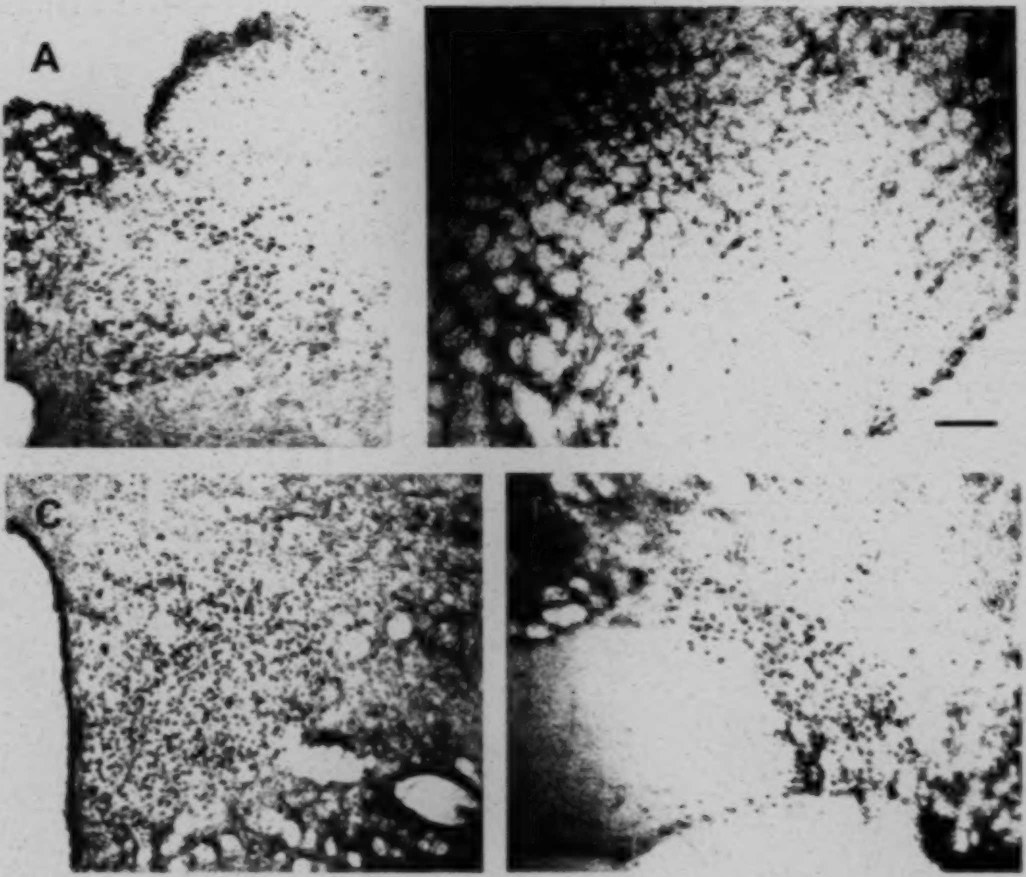


Figure 3-2. Representative photomicrographs showing immunohistochemical labeling of Fos in transverse sections of the (A) NTS, (B) VLM, (C) PVN, and (D) SON of neurally intact, splenic vein-occluded animals. Fos is the protein product of *c-fos*, an immediate-early gene transcribed within minutes of a physiological or pathophysiological stimulus. Fos is visualized as a brown reaction product after immunohistochemical staining procedures. Localization of Fos was similar for control animals, and for renal-denervated and renal + splenic-denervated animals (not shown). Scale bar = 50 µm (A-D).

Table 3-1. Baseline values of SVP and MAP from control and experimental intact, renal-denervated, and renal + splenic-denervated animals.

Experimental Group		*n*	SVP (mmHg)	MAP (mmHg)
Intact	Control	14	14.2 ± 2.0	105.1 ± 3.4
	SVO	6	9.3 ± 1.4	115.7 ± 1.7
RD	Control	7	16.4 ± 1.4	100.6 ± 2.3
	SVO	6	13.3 ± 2.1	105.9 ± 7.1
RSD	Control	6	10.4 ± 1.5	110.4 ± 4.4
	SVO	4	9.8 ± 0.3	108.7 ± 7.1

Values are presented as means ± SE. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated; Control, non-occluded animals; SVO, splenic vein-occluded animals; MAP, mean arterial pressure; SVP, splenic venous pressure; *n* = number.

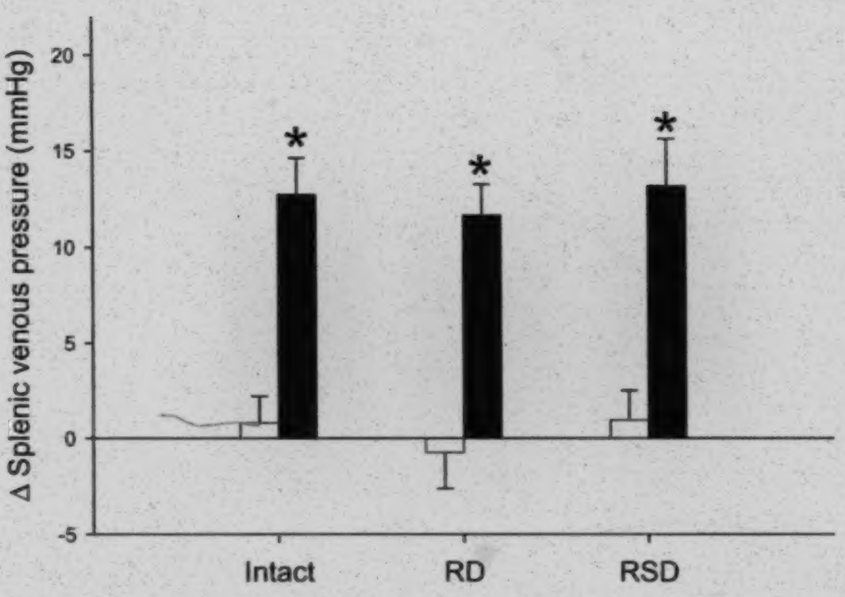


Figure 3-3. Change in SVP from baseline in control (open bars) and splenic vein-occluded (solid bars) animals. Data represent an average of 5 minutes ~30 minutes into the occlusion period. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated. Vertical bars delineate SE of the mean. *Significant difference from control counterparts. $P < 0.05$.

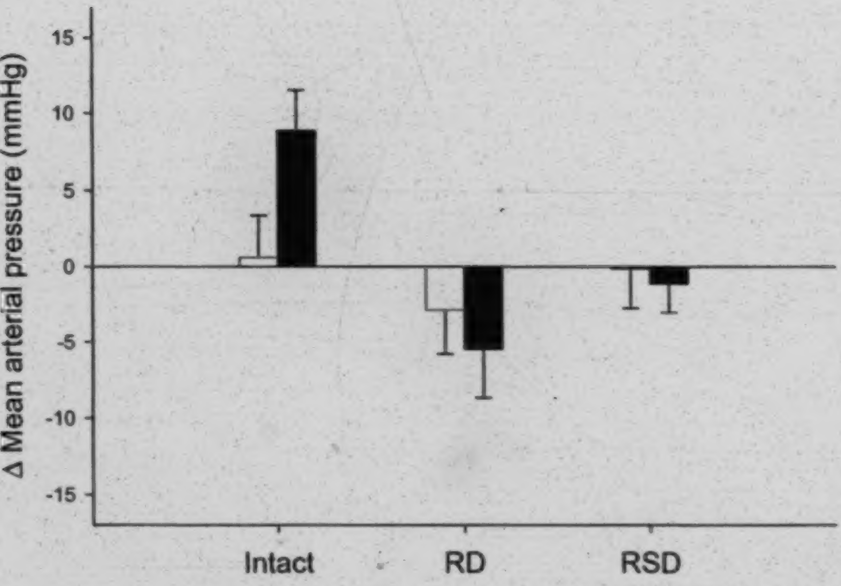


Figure 3-4. Change in mean arterial pressure (MAP) from baseline in control (open bars) and splenic vein-occluded (solid bars) animals. Data represent an average of 5 minutes ~30 minutes into the occlusion period. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated. Vertical bars delineate SE of the mean.

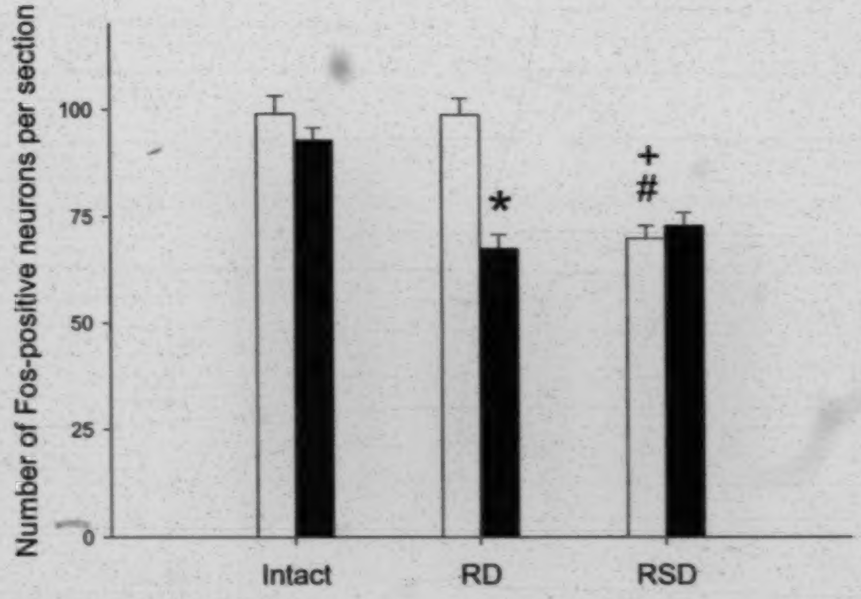


Figure 3-5. Numbers of neurons labeled for Fos present in the nucleus of the tractus solitarius (NTS) in control (open bars) and splenic vein-occluded (solid bars) rats. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated. Vertical bars delineate SE of the mean. *Significant difference from control within the same group; # significant difference from intact controls; + significant difference from RD controls. $P < 0.05$.

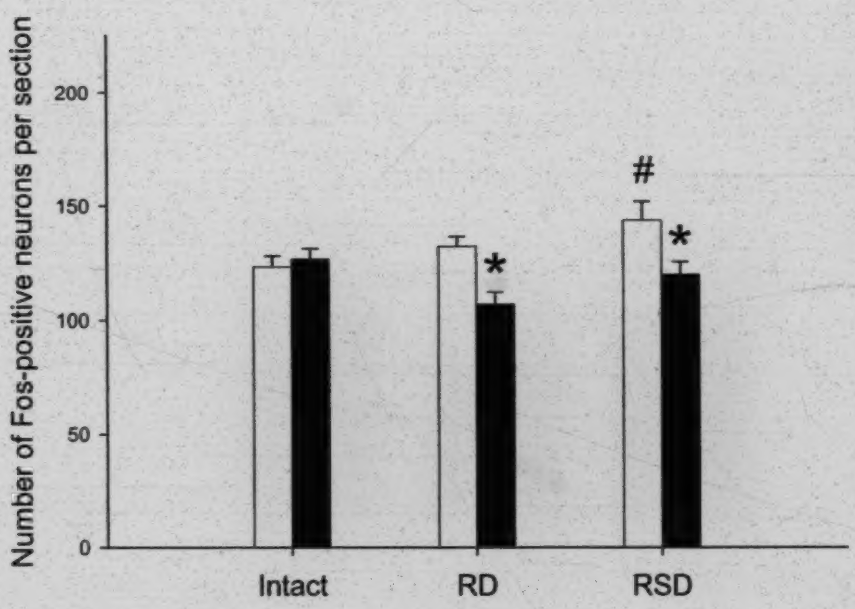


Figure 3-6. Numbers of Fos-positive neurons present in the ventrolateral medulla (VLM) in control (open bars) and splenic vein-occluded (solid bars) rats. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated. Vertical bars delineate SE of the mean. *Significant difference from control within the same group; # significant difference from intact controls; + significant difference from RD controls. $P < 0.05$.

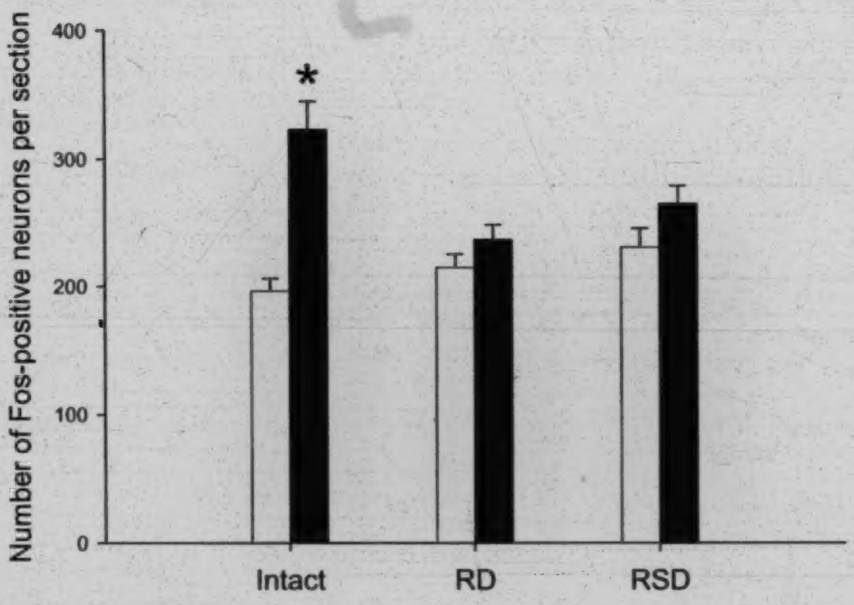


Figure 3-7*A*. Numbers of neurons labeled for Fos in the parvocellular (PC) region of the paraventricular nucleus (PVN) in control (open bars) and splenic vein-occluded (solid bars) rats. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated. Vertical bars delineate SE of the mean. *Significant difference from control within the same group. $P < 0.05$.

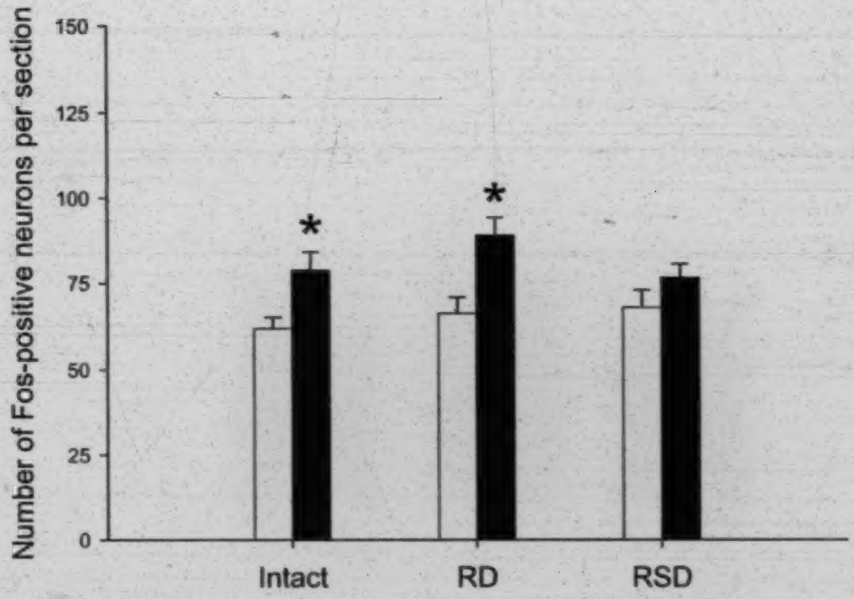


Figure 3-7*B*. Numbers of Fos-positive neurons present in the magnocellular (MC) region of the paraventricular nucleus (PVN) in control (open bars) and splenic vein-occluded (solid bars) rats. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated. Vertical bars delineate SE of the mean. *Significant difference from control within the same group. $P < 0.05$.

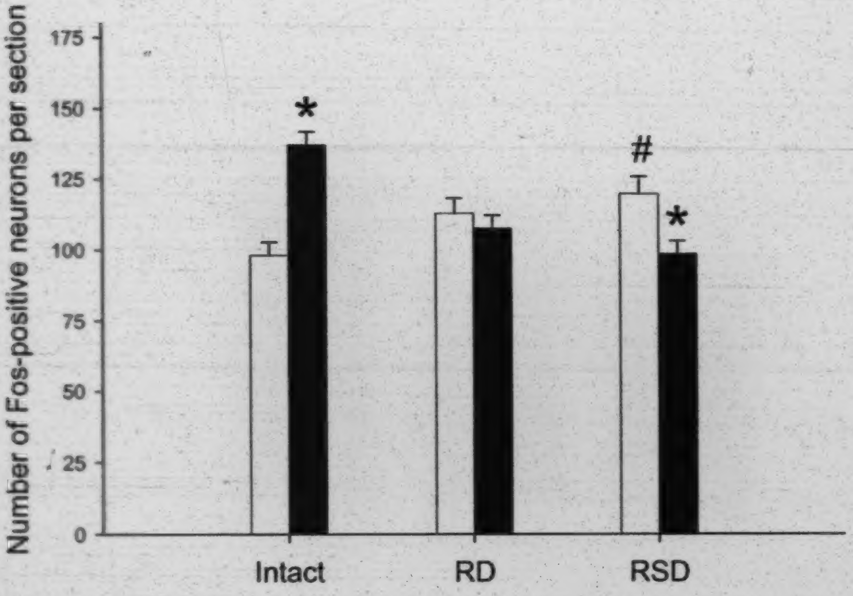


Figure 3-8. Numbers of neurons labeled for Fos in the supraoptic nucleus (SON) of the hypothalamus in control (open bars) and splenic vein-occluded (solid bars) rats. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated. Vertical bars delineate SE of the mean. *Significant difference from control within the same group; # significant difference from intact controls. $P < 0.05$.

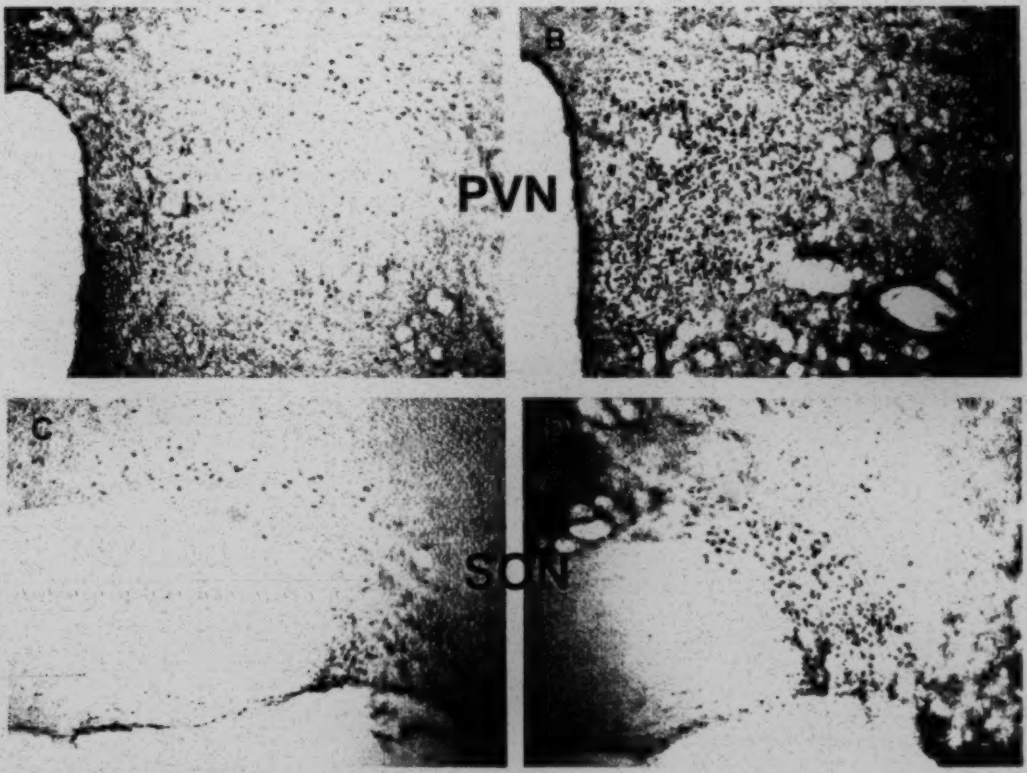


Figure 3-9. Representative photomicrographs showing immunohistochemical labeling of Fos in transverse sections of the hypothalamus in neurally intact non-occluded controls (A and C) and splenic vein-occluded (B and D) animals. The numbers of labeled neurons in the paraventricular nucleus (PVN, A vs. B) and supraoptic nucleus (SON, C vs. D) are shown to significantly increase in response to splenic vein occlusion. Scale bar = 50 μm (A-D).

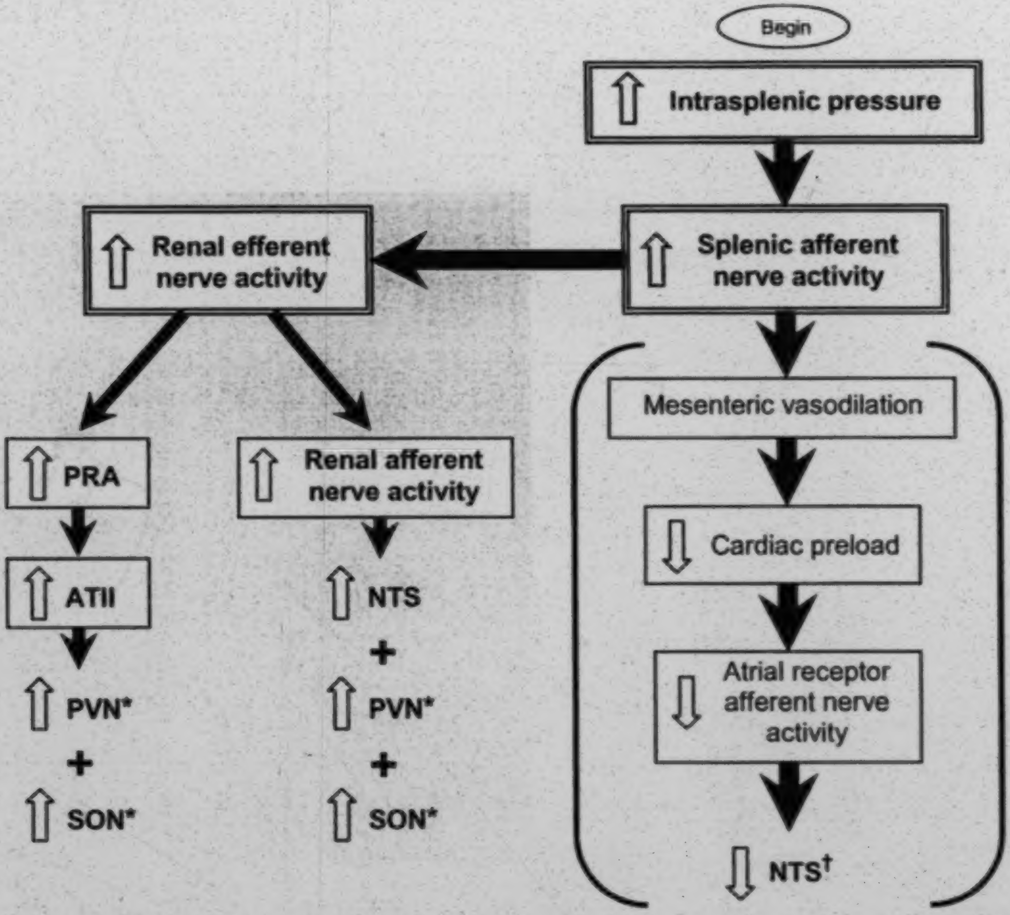
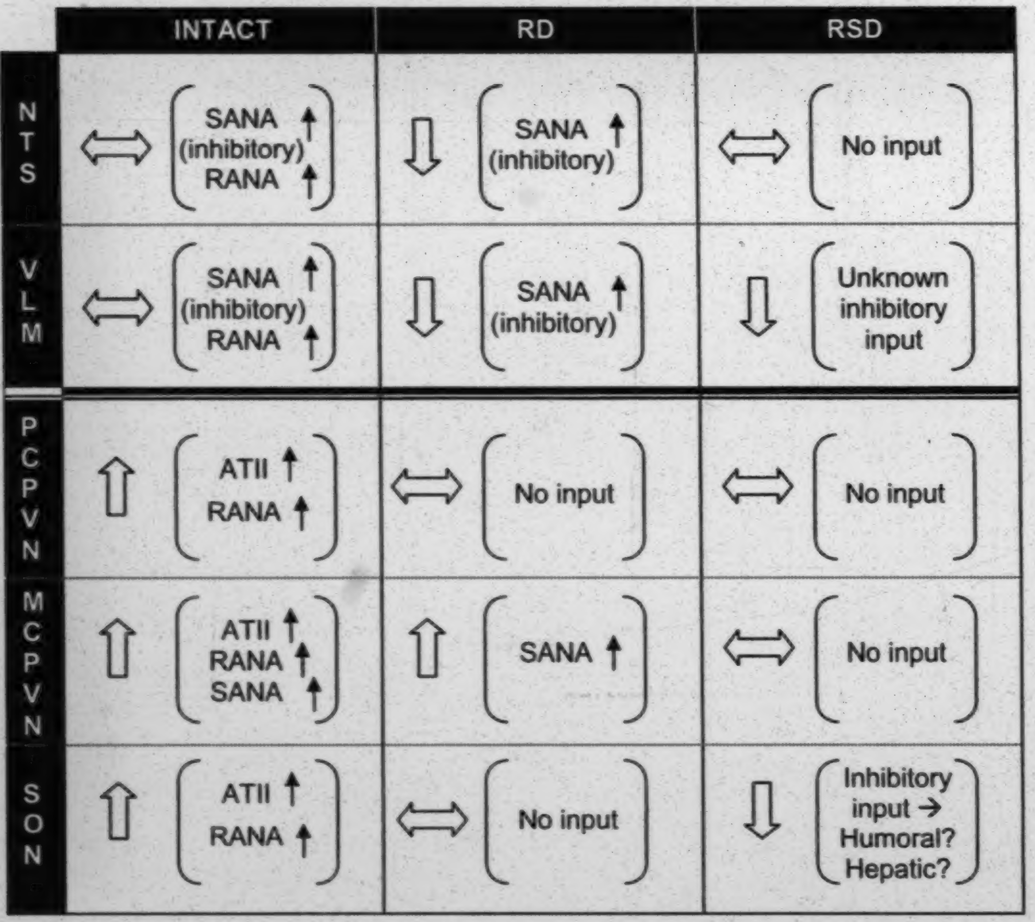

Begin
⇧ Intrasplenic pressure
⇧ Renal efferent nerve activity
⇧ Splenic afferent nerve activity
⇧ PRA
⇧ Renal afferent nerve activity
⇧ ATII
⇧ NTS
+
⇧ PVN*
+
⇧ SON*
⇧ PVN*
+
⇧ SON*
Mesenteric vasodilation
⇩ Cardiac preload
⇩ Atrial receptor afferent nerve activity
⇩ NTS†

Figure 3-10. Flowchart illustrating the expected and proposed central neural effects of the splenorenal reflex, as caused by an increase in splenic afferent nerve activity and renal efferent nerve activity. Central effects expected to arise from the kidney are shown in the shaded area (left); * represents observations of the present study as seen in intact animals. The bracketed area (right) refers to a proposed mechanism by which an increase in splenic afferent nerve activity may cause a reduction in neuronal activity in the NTS; † represents observations of the present study as seen in renal-denervated animals. Abbreviations: PRA, plasma renin activity; ATII, angiotensin II; NTS, nucleus of the tractus solitarius; PVN, paraventricular nucleus; SON, supraoptic nucleus.



	INTACT	RD	RSD
NTS	⇔ SANA ↑ (inhibitory) RANA ↑	⇩ SANA ↑ (inhibitory)	⇔ No input
VLM	⇔ SANA ↑ (inhibitory) RANA ↑	⇩ SANA ↑ (inhibitory)	⇩ Unknown inhibitory input
PCPVN	⇧ ATII ↑ RANA ↑	⇔ No input	⇔ No input
MCPVN	⇧ ATII ↑ RANA ↑ SANA ↑	⇧ SANA ↑	⇔ No input
SON	⇧ ATII ↑ RANA ↑	⇔ No input	⇩ Inhibitory input → Humoral? Hepatic?

Figure 3-11. Summary of effects on activation of brainstem (NTS, VLM) and hypothalamic (PVN, SON) nuclei in response to splenic vein occlusion (SVO) in intact and denervated animals. Block arrows refer to the observed effect of SVO (vs. control animals) on numbers of neurons labeled for Fos. Proposed reasoning for observed changes in Fos labeling is shown in bracketed areas. Abbreviations: RD, renal-denervated; RSD, renal + splenic-denervated; NTS, nucleus of the tractus solitarius; VLM, ventrolateral medulla; PC PVN, parvocellular region of the paraventricular nucleus; MC PVN, magnocellular region of the paraventricular nucleus; SON, supraoptic nucleus; SANA, splenic afferent nerve activity; RANA, renal afferent nerve activity; ATII, angiotensin II.

BIBLIOGRAPHY

1. Ammons, W. S. Bowditch Lecture. Renal afferent inputs to ascending spinal pathways. *Am J Physiol* 262: R165-76, 1992.

2. Andrew, P., Y. Deng, and S. Kaufman. Fluid extravasation from spleen reduces blood volume in endotoxemia. *Am J Physiol Regul Integr Comp Physiol* 278: R60-5, 2000.

3. Andrew, P. S. , Y. Deng, R. Sultanian, and S. Kaufman. Nitric oxide increases fluid extravasation from the splenic circulation of the rat. *Am J Physiol Regul Integr Comp Physiol* 280: R959-67, 2001.

4. Badoer, E., M. J. McKinley, B. J. Oldfield, and R. M. McAllen. Distribution of hypothalamic, medullary and lamina terminalis neurons expressing Fos after hemorrhage in conscious rats. *Brain Res* 582: 323-8, 1992.

5. Badoer, E., M. J. McKinley, B. J. Oldfield, and R. M. McAllen. A comparison of hypotensive and non-hypotensive hemorrhage on Fos expression in spinally projecting neurons of the paraventricular nucleus and rostral ventrolateral medulla. *Brain Res* 610: 216-23, 1993.

6. Badoer, E., B. J. Oldfield, and M. J. McKinley. Haemorrhage-induced production of Fos in neurons of the lamina terminalis: role of endogenous angiotensin II. *Neurosci Lett* 159: 151-4, 1993.

7. Breitman, D. R. and S. S. Lee. Blunted responsiveness of the neuronal activation marker Fos in brainstem cardiovascular nuclei of cirrhotic rats. *Hepatology* 26: 1380-5, 1997.

8. Calaresu, F. R., J. C. Tobey, S. R. Heidemann, and L. C. Weaver. Splenic and renal sympathetic responses to stimulation of splenic receptors in cats. *Am J Physiol* 247: R856-65, 1984.

9. Caverson, M. M. and J. Ciriello. Effect of stimulation of afferent renal nerves on plasma levels of vasopressin. *Am J Physiol* 252: R801-7, 1987.

10. Caverson, M. M. and J. Ciriello. Contribution of paraventricular nucleus to afferent renal nerve pressor response. *Am J Physiol* 254: R531-43, 1988.

11. Caverson, M. M. and J. Ciriello. Renal and cardiovascular afferent inputs to hypothalamic paraventriculo-spinal neurons. *Neurosci Lett* 95: 167-72, 1988.

12. Caverson, M. M., J. Ciriello, and F. R. Calaresu. Cardiovascular afferent inputs to neurons in the ventrolateral medulla projecting directly to the central autonomic area of the thoracic cord in the cat. *Brain Res* 274: 354-8, 1983.

13. Caverson, M. M., J. Ciriello, and F. R. Calaresu. Direct pathway from cardiovascular neurons in the ventrolateral medulla to the region of the intermediolateral nucleus of the upper thoracic cord: an anatomical and electrophysiological investigation in the cat. *J Auton Nerv Syst* 9: 451-75, 1983.

14. Chen, A. and S. Kaufman. Splenic blood flow and fluid efflux from the intravascular space in the rat. *J Physiol* 490 (Pt 2): 493-9, 1996.

15. Ciriello, J. and F. R. Calaresu. Central projections of afferent renal fibers in the rat: an anterograde transport study of horseradish peroxidase. *J Auton Nerv Syst* 8: 273-85, 1983.

16. Ciriello, J. and M. M. Caverson. Direct pathway from neurons in the ventrolateral medulla relaying cardiovascular afferent information to the supraoptic nucleus in the cat. *Brain Res* 292: 221-8, 1984.

17. Dampney, R. A., J. Czachurski, K. Dembowsky, A. K. Goodchild, and H. Seller. Afferent connections and spinal projections of the pressor region in the rostral ventrolateral medulla of the cat. *J Auton Nerv Syst* 20: 73-86, 1987.

18. Dampney, R. A. and Horiuchi, J. Functional organisation of central cardiovascular pathways: studies using c-fos gene expression. *Prog Neurobiol* 71: 359-84, 2003.

19. Dawson, C. A., J. H. Jhamandas, and T. L. Krukoff. Activation by systemic angiotensin II of neurochemically identified neurons in rat hypothalamic paraventricular nucleus. *J Neuroendocrinol* 10: 453-9, 1998.

20. Day, T. A. and J. Ciriello. Afferent renal nerve stimulation excites supraoptic vasopressin neurons. *Am J Physiol* 249: R368-71, 1985.

21. Day, T. A. and J. Ciriello. Effects of renal receptor activation on neurosecretory vasopressin cells. *Am J Physiol* 253: R234-41, 1987.

22. de Vries, A., J. F. Paton, S. L. Lightman, and C. A. Lowry. Characterisation of c-Fos expression in the central nervous system of mice following right atrial injections of the 5-HT3 receptor agonist phenylbiguanide. *Auton Neurosci* 123: 62-75, 2005.

23. Deng, Y. and S. Kaufman. Splenorenal reflex regulation of arterial pressure. *Hypertension* 38: 348-52, 2001.

24. Dragunow, M. and R. Faull. The use of c-fos as a metabolic marker in neuronal pathway tracing. *J Neurosci Methods* 29: 261-5, 1989.

25. Fitch, G. K., K. P. Patel, and M. L. Weiss. Increased renal interstitial hydrostatic pressure causes c-fos expression in the rat's spinal cord dorsal horn. *Brain Res* 753: 340-7, 1997.

26. Fitch, G. K., K. P. Patel, and M. L. Weiss. Activation of renal afferent pathways following furosemide treatment. I. Effects Of survival time and renal denervation. *Brain Res* 861: 363-76, 2000.

27. Fitch, G. K. and M. L. Weiss. Ureteral ligation induces Fos expression in the dorsal horn. *Brain Res* 723: 199-205, 1996.

28. Gattone, V. H. 2nd, C. F. Marfurt, and S. Dallie. Extrinsic innervation of the rat

kidney: a retrograde tracing study. *Am J Physiol* 250: F189-96, 1986.

29. Greenway, C. V. Role of splanchnic venous system in overall cardiovascular homeostasis. *Fed Proc* 42: 1678-84, 1983.

30. Hamza, S. M. and S. Kaufman. Splenorenal reflex modulates renal blood flow in the rat. *J Physiol* 558: 277-82, 2004.

31. Hayes, K., C. P. Yardley, and L. C. Weaver. Evidence for descending tonic inhibition specifically affecting sympathetic pathways to the kidney in rats. *J Physiol* 434: 295-306, 1991.

32. Herbert, J., M. L. Forsling, S. R. Howes, P. M. Stacey, and H. M. Shiers. Regional expression of c-fos antigen in the basal forebrain following intraventricular infusions of angiotensin and its modulation by drinking either water or saline. *Neuroscience* 51: 867-82, 1992.

33. Herman, N. L., D. R. Kostreva, and J. P. Kampine. Splenic afferents and some of their reflex responses. *Am J Physiol* 242: R247-54, 1982.

34. Horiuchi, J., P. D. Potts, J. W. Polson, and R. A. Dampney. Distribution of neurons projecting to the rostral ventrolateral medullary pressor region that are activated by sustained hypotension. *Neuroscience* 89: 1319-29, 1999.

35. Jacob, F., B. G. LaBine, P. Ariza, S. A. Katz, and J. W. Osborn. Renal denervation

causes chronic hypotension in rats: role of beta1-adrenoceptor activity. *Clin Exp Pharmacol Physiol* 32: 255-62, 2005.

36. Jurzik, L., M. Froh, R. H. Straub, J. Scholmerich, and R. Wiest. Up-regulation of nNOS and associated increase in nitrergic vasodilation in superior mesenteric arteries in pre-hepatic portal hypertension. *J Hepatol* 43: 258-65, 2005.

37. Kaufman, S. Role of spleen in ANF-induced reduction in plasma volume. *Can J Physiol Pharmacol* 70: 1104-8, 1992.

38. Kaufman, S., P. Andrew, R. Sultanian, and Y. Deng. Adrenomedullin increases fluid extravasation from the splenic circulation of the rat. *J Physiol* 534: 527-33, 2001.

39. Kaufman, S. and Y. Deng. Splenic control of intravascular volume in the rat. *J Physiol* 468: 557-65, 1993.

40. Kopp, U. C. and G. F. DiBona. Interaction between neural and nonneural mechanisms controlling renin secretion rate. *Am J Physiol* 246: F620-6, 1984.

41. Kopp, U. C., L. A. Olson, and G. F. DiBona. Renorenal reflex responses to mechano- and chemoreceptor stimulation in the dog and rat. *Am J Physiol* 246: 67-77, 1984.

42. Krukoff, T. L., D. MacTavish, K. H. Harris, and J. H. Jhamandas. Changes in blood volume and pressure induce c-fos expression in brainstem neurons that project to the paraventricular nucleus of the hypothalamus. *Brain Res Mol Brain Res* 34: 99-108,

1995.

43. Lebrun, C. J., A. Blume, T. Herdegen, K. Seifert, R. Bravo, and T. Unger. Angiotensin II induces a complex activation of transcription factors in the rat brain: expression of Fos, Jun and Krox proteins. *Neuroscience* 65: 93-9, 1995.

44. Lind, R. W., L. W. Swanson, and D. Ganten. Organization of angiotensin II immunoreactive cells and fibers in the rat central nervous system. An immunohistochemical study. *Neuroendocrinology* 40: 2-24, 1985.

45. Lundgren, O. Role of splanchnic resistance vessels in overall cardiovascular homeostasis. *Fed Proc* 42: 1673-7, 1983.

46. McKinley, M. J., E. Badoer, L. Vivas, and B. J. Oldfield. Comparison of c-fos expression in the lamina terminalis of conscious rats after intravenous or intracerebroventricular angiotensin. *Brain Res Bull* 37: 131-7, 1995.

47. Meckler, R. L. and L. C. Weaver. Persistent firing of splenic and renal afferent nerves after acute decentralization but failure to produce ganglionic reflexes. *Neurosci Lett* 88: 167-72, 1988.

48. Miselis, R. R. The efferent projections of the subfornical organ of the rat: a circumventricular organ within a neural network subserving water balance. *Brain Res* 230: 1-23, 1981.

49. Moll-Kaufmann, C., L. T. Sumanovski, and C. C. Sieber. Neurally-mediated vasodilatation in normal and portal hypertensive rats: role of nitric oxide and calcitonin gene-related peptide. *J Hepatol* 28: 1031-6, 1998.

50. Moncrief, K. and S. Kaufman. Splenic baroreceptors control splenic afferent nerve activity. *Am J Physiol Regul Integr Comp Physiol* 290: R352-6, 2006.

51. Morgan, J. I., D. R. Cohen, J. L. Hempstead, and T. Curran. Mapping patterns of c-fos expression in the central nervous system after seizure. *Science* 237: 192-7, 1987.

52. Moss, N. G. Renal function and renal afferent and efferent nerve activity. *Am J Physiol* 243: F425-33, 1982.

53. Nance, D. M. and J. Burns. Innervation of the spleen in the rat: Evidence for absence of afferent innervation. *Brain Behav Immun* 3: 281-90, 1989.

54. Nilaver, G., E. A. Zimmerman, J. Wilkins, J. Michaels, D. Hoffman, and A. J. Silverman. Magnocellular hypothalamic projections to the lower brain stem and spinal cord of the rat. Immunocytochemical evidence for predominance of the oxytocin-neurophysin system compared to the vasopressin-neurophysin system. *Neuroendocrinology* 30: 150-8, 1980.

55. Oldfield, B. J. and M. J. McKinley. Distribution of Fos in rat brain resulting from endogenously-generated angiotensin II. *Kidney Int* 46: 1567-9, 1994.

56. Paton, J. F. Pattern of cardiorespiratory afferent convergence to solitary tract neurons driven by pulmonary vagal C-fiber stimulation in the mouse. *J Neurophysiol* 79: 2365-73, 1998.

57. In Paxinos, G. and C. Watson, eds. The Rat Brain in Sterotaxic Coordinates. San Diego, CA, USA, Academic Press Inc. 1986.

58. Potts, P. D., Y. Hirooka, and R. A. Dampney. Activation of brain neurons by circulating angiotensin II: direct effects and baroreceptor-mediated secondary effects. *Neuroscience* 90: 581-94, 1999.

59. Recordati, G., N. G. Moss, S. Genovesi, and P. Rogenes. Renal chemoreceptors. *J Auton Nerv Syst* 3: 237-51, 1981.

60. Recordati, G. M., N. G. Moss, and L. Waselkov. Renal chemoreceptors in the rat. *Circ Res* 43: 534-43, 1978.

61. Rivest, S. and N. Laflamme. Neuronal activity and neuropeptide gene transcription in the brains of immune-challenged rats. *J Neuroendocrinol* 7: 501-25, 1995.

62. Rivest, S., N. Laflamme, and R. E. Nappi. Immune challenge and immobilization stress induce transcription of the gene encoding the CRF receptor in selective nuclei of the rat hypothalamus. *J Neurosci* 15: 2680-95, 1995.

63. Ross, C. A., D. A. Ruggiero, and D. J. Reis. Afferent projections to cardiovascular

portions of the nucleus of the tractus solitarius in the rat. *Brain Res* 223: 402-8, 1981.

64. Rowland, N. E., B. H. Li, A. K. Rozelle, M. J. Fregly, M. Garcia, and G. C. Smith. Localization of changes in immediate early genes in brain in relation to hydromineral balance: intravenous angiotensin II. *Brain Res Bull* 33: 427-36, 1994.

65. Sagar, S. M., F. R. Sharp, and T. Curran. Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* 240: 1328-31, 1988.

66. Sawchenko, P. E. and L. W. Swanson. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* 205: 260-72, 1982.

67. Sawchenko, P. E. and L. W. Swanson. The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain Res* 257: 275-325, 1982.

68. Sawchenko, P. E., L. W. Swanson, H. W. Steinbusch, and A. A. Verhofstad. The distribution and cells of origin of serotonergic inputs to the paraventricular and supraoptic nuclei of the rat. *Brain Res* 277: 355-60, 1983.

69. Simon, O. R. and L. P. Schramm. The spinal course and medullary termination of myelinated renal afferents in the rat. *Brain Res* 290: 239-47, 1984.

70. Song, D., K. A. Sharkey, D. R. Breitman, Y. Zhang, and S. S. Lee. Disordered central

cardiovascular regulation in portal hypertensive and cirrhotic rats. *Am J Physiol Gastrointest Liver Physiol* 280: G420-30, 2001.

71. Stella, A. The kidney as a sensor: functional evidence. *J Hypertens Suppl* 10: S113-9, 1992.

72. Sun, M. K. Central neural organization and control of sympathetic nervous system in mammals. *Prog Neurobiol* 47: 157-233, 1995.

73. Swanson, L. W. and Kuypers, H. G. The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J Comp Neurol* 194(3): 555-70, 1980.

74. Swanson, L. W. and Sawchenko, P. E. Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* 31(6): 410-7, 1980.

75. Swanson, L. W., P. E. Sawchenko, A. Berod, B. K. Hartman, K. B. Helle, and D. E. Vanorden. An immunohistochemical study of the organization of catecholaminergic cells and terminal fields in the paraventricular and supraoptic nuclei of the hypothalamus. *J Comp Neurol* 196: 271-85, 1981.

76. Vorobioff, J., J. E. Bredfeldt, and R. J. Groszmann. Hyperdynamic circulation in portal-hypertensive rat model: a primary factor for maintenance of chronic portal

hypertension. *Am J Physiol* 244: G52-7, 1983.

77. Weiss, M. L. and G. I. Hatton. Collateral input to the paraventricular and supraoptic nuclei in rat. I. Afferents from the subfornical organ and the anteroventral third ventricle region. *Brain Res Bull* 24: 231-8, 1990.

78. Wiest, R., L. Jurzik, L. Moleda, M. Froh, B. Schnabl, S. von Horsten, J. Scholmerich, and R. H. Straub. Enhanced Y1-receptor-mediated vasoconstrictive action of neuropeptide Y (NPY) in superior mesenteric arteries in portal hypertension. *J Hepatol* 44: 512-9, 2006.

79. Wilkin, L. D., L. D. Mitchell, D. Ganten, and A. K. Johnson. The supraoptic nucleus: afferents from areas involved in control of body fluid homeostasis. *Neuroscience* 28: 573-84, 1989.

80. Xia, Y. and T. L. Krukoff. Differential neuronal activation in the hypothalamic paraventricular nucleus and autonomic/neuroendocrine responses to I.C.V. endotoxin. *Neuroscience* 121: 219-31, 2003.

81. Zardetto-Smith, A. M., R. L. Thunhorst, M. Z. Cicha, and A. K. Johnson. Afferent signaling and forebrain mechanisms in the behavioral control of extracellular fluid volume. *Ann N Y Acad Sci* 689: 161-76, 1993.

CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

4.1. DISCUSSION

The value of the spleen for human survival has, historically, been widely underestimated. This is evidenced by the routine frequency with which the spleen has been surgically removed in cases of trauma or disease (30). Although the medical and scientific communities have begun to appreciate the importance of the spleen in terms of its immunological functions (30) and the consequences of its removal in those regards, the potential to which the spleen may influence the cardiovascular system has received much less attention.

The spleen is part of the splanchnic circulation, comprising the gastric, small intestinal, colonic, pancreatic, hepatic and splenic circulations (34). The splanchnic circulation is well established as a major contributor to cardiovascular homeostasis through its role as a blood reservoir, providing most of the body's capacitance response to hypotension (16,34,35). Sympathetic stimulation can mobilize up to 27% of total blood volume from the splanchnic venous circulation, serving as an excellent means of redistributing blood volume throughout the cardiovascular system (14).

We have already identified a reflex pathway in which the spleen and the kidneys interact in regulating blood pressure and blood volume, known as the splenorenal reflex (9). The existence of other reflexes by which the spleen may affect cardiovascular regulation are indicated by studies which stimulate the splenic nerves. There appears to be a bidirectional connection between the splenic nerves and the heart. Stimulation of splenic afferents is known to have a cardiostimulatory effect (4,17); conversely, unloading of cardiac receptors has been shown to excite splenic nerves (22). This suggests a neural mechanism by which the spleen and the heart coordinate information on blood volume to regulate fluid homeostasis; cardiac receptors signal central blood volume and splenic receptors signal the level of splanchnic pooling. Both have neural connections with the kidneys, providing an effective control on fluid output.

In addition, an increase in splenic afferent nerve activity has been shown to elicit reflex increases in splenic efferent nerve activity, ie. spleno-splenic reflex (4). In pathophysiological conditions such as portal hypertension, this would suggest a mechanism by which the spleen could increase fluid extravasation to reduce plasma volume, thereby reducing splanchnic congestion and portal pressure. Studies have shown that, in humans, portal hypertension is exacerbated by splenectomy (27,33). This has been found in the rat as well, with a study demonstrating that splenectomy increases portal pressure (26). These studies suggest that the spleen does indeed play a role in relieving venous congestion in portal hypertension through fluid efflux. This also implicates the neural circuitry of the spleen in normal regulation of intravascular volume.

Evidence suggests that sensory afferent fibers from the spleen release a peptidergic neurotransmitter(s), such as substance P, neuropeptide Y (NPY) and/or an opiate-like peptide (6,12,28,29,31,32,38). In addition, the mammalian spleen has been shown to contain a natriuretic, hypotensive factor that may play a role in cardiorenal homeostasis by triggering a primary decrease in plasma volume and a delayed increase in urine output (8,21). A proposed mechanism by which splenic factor achieves this diuretic effect is through selective vasodilation of the afferent glomerular arteriole and constriction of the efferent glomerular arteriole of the kidney (21). The effects of splenic factor closely resemble those of atrial natriuretic factor (ANF) (21), which is known to increase hematocrit and reduce plasma volume (1,7,10,24). The spleen is believed to be a major site of ANF-induced fluid extravasation, through differential vascular tone of the splenic resistance arteries and veins [illegible] intrasplenic capillary hydrostatic pressure (2,3,19,20,37). Since the spleen is freely permeable to plasma proteins, ultrafiltration of iso-oncotic fluid from the splenic circulation directly influences plasma volume and blood pressure (5).

The novel findings of the presented work suggest a role for the splenic afferent nerves in central modulation of cardiovascular regulation. It had originally been hypothesized that the splenorenal reflex may act through the brain, because electrical stimulation of splenic afferent nerves was known to inhibit rostral VLM neurons (13). On the other hand, this reflex had been identified as a purely spinal reflex pathway (32). Furthermore, reflex cardiovascular responses elicited by electrical stimulation of splenic afferent fibers persisted after sectioning of both vagi and phrenic nerves (17), suggesting that splenic sensory afferents do not travel to the CNS with the vagus or phrenic nerves. The results presented in Chapter 3 lend support to a lack of direct splenic afferent input to the brainstem, although there is evidence for direct input to the magnocellular region of the PVN. It appears as though activation of the splenorenal reflex does influence central cardiovascular pathways, with the majority of the effects arising secondarily to renal responses. Furthermore, it was suggested that an important influence of splenic afferent nerves may be through a putative spleno-intestinal reflex, which is implicated in exacerbating the splanchnic vasodilation and pathogenesis of portal hypertension.

In this regard, it is of interest that a study examining central cardiovascular mechanisms underlying the development of hyperdynamic circulation points to neural activation as a permissive factor in the pathogenesis of portal hypertension (36). They found that the onset of CNS effects in portal vein stenosis preceded the onset of cardiovascular anomalies, indicating that activation of the *c-fos* pathway triggered circulatory changes leading to the development of a hyperdynamic circulation (36). Furthermore, these authors had previously shown that capsaicin ablation of sensory afferent nerves abolished changes in Fos expression in the brainstem and later development of hyperdynamic circulation (25), suggesting that sensory afferents served as the link between the peripheral vasculature and CNS activation. The results presented in this dissertation implicate the spleen and splenic afferents as a potential source for mediating the central cardiovascular dysfunction contributing to the pathogenesis of portal hypertension.

4.2. METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS

It is important to consider the model being used to study physiological and pathophysiological processes. First and foremost, we study *in vivo* physiological manipulations in acute and chronic rat preparations. There are a number of ethical issues to consider in utilizing whole-animal studies of this nature. Although there are alternatives to animal use, such as mathematical models or cell culture, they cannot answer all of our scientific inquiries. Although not without its own limitations, studying *in vivo* integrative physiology more closely approximates that which would be expected in the human situation. Cell culture systems and *in situ* studies are limited in that they represent tissues taken out of the body, which may not behave naturally when removed from the blood, immune system, nervous system, and from surrounding complementary tissues. While human studies best represent the clinical scenario, human patients can be exceptionally difficult to control, with issues of non-compliance and/or false reporting leading to erroneous conclusions. Furthermore, the natural course of a disease in a human patient may take many years, making it extremely difficult to study, compared to using a rat, for example, in which the disease may progress over a few weeks or better yet, may be induced and/or simulated in an acute study. In our studies, we are able to mimic the effects of portal hypertension in an acute scenario using portal vein ligation and/or splenic vein occlusion, thereby minimizing the time required to answer our scientific questions.

In vivo studies are not without their own complications however. There is a great deal of uncertainty with whole animal studies, with an exponential number of variables introduced to the experiment that could otherwise be controlled in an isolated study. The use of control experiments becomes exceptionally important in dealing with whole animal studies. Although a great deal of variability exists between individual animal subjects, appropriate controls will account for that variability in order to maintain consistency across experimental groups. However, reproducibility can still be difficult when such large variations exist and success rates rarely reach 100%. With difficult surgical procedures, unexpected complications may hinder production of meaningful results, at the expense of valuable resources.

We use the Long Evans breed of laboratory rat for our experiments. The rat is an ideal model in examining the physiology of the spleen because it is qualitatively similar to the human (15). Both the human and rat spleen have limited storage capacity; the rat has a non-contractile outer capsule which limits its ability to expel high hematocrit blood in times of need, such as hemorrhage, thus eliminating one variable, namely the effects of an autologous blood transfusion. Furthermore, rats have the same elaborate pathways for blood flow as humans, and red blood cell washout tests show that the rat spleen is indeed similar to humans (15).

The experiments described in Chapter 2 were designed to distinguish between the effects on splenic afferent nerve activity of changing blood pressure and changing blood flow. Both flow and pressure changed in our experimental groups (venous occlusion vs. arterial occlusion), but by creating the same reduction in blood flow between the groups, we were able to eliminate the change in flow as a variable in our experiment. Ideally, there would have been only two experimental groups, venous-occluded and arterial-occluded, in which we could have examined the simultaneous effects of partial splenic artery occlusion on both venous and arterial blood flows (as was carried out in the venous-occluded group). However, due to the limited space within the abdominal cavity of the rat, the anatomical arrangement of the splenic circulation dictating our balloon occluder placement, and the subsequent confined arrangement of recording equipment, we were unable to "fit" both flow probes on the artery and vein in the artery-occluded group. As such, we subdivided the splenic artery-occluded group into arterial flow-matched (SAO_{AF}) and venous flow-matched (SAO_{VF}) animals. The limitation of subdividing this group was that we possibly introduced variation between the two subgroups. The degree of artery occlusion may have differed in order to match the reduction in either venous or arterial blood flow. However, because there were no significant differences between our arterial occluded groups, we can assume that the variation in degree of vessel occlusion was negligible, and our groups were, in effect, treated equally except for physical placement of the flow probe.

We were also limited by the inability to measure splenic arterial pressure in our arterial-occluded groups. The placement of the balloon downstream (distal) to the gastric artery made arterial pressure measurement impossible. This hemodynamic measurement would have been useful in verifying the degree of arterial occlusion between venous flow-matched and arterial flow-matched SAO groups, while also confirming the reduction in splenic arterial pressure assumed with occlusion of that vessel. Alternatively, the experiments could have been conducted using a measure of *intrasplenic* pressure rather than splenic venous and arterial pressures, although these alternative methods pose their own set of limitations.

A difficulty was encountered in simultaneously measuring blood flow and extracellular nerve activity in the experiments of Chapter 2. Mineral oil was required to electrically isolate the nerve and maintain nerve viability for the duration of the experiment. Normally, a more viscous acoustic couplant is optimal for obtaining strong flow readings with the ultrasonic flow probes. Although cautiously approved for use in mineral oil by Transonic technicians, the flow probes proved to be exceptionally sensitive to minor vessel and/or couplant movements, such that signal drop-out was a frequent occurrence. A more viscous couplant suitable for both extracellular nerve recording and ultrasonic flow measurement, if available, would have improved the success rate of experiments and may have produced stronger (ie. higher) flow readings for splenic inflow and outflow.

The experiments presented in Chapter 3 produced interesting and significant albeit somewhat ambiguous results. The limitations of this study are also addressed in the subsequent section on *Possible Future Studies*. The study was designed with a simple objective in mind – to investigate the effect of splenic vein occlusion on the central response to splenic baroreceptor (and splenorenal reflex) activation, using localization of *c-fos* expression in neurons of known cardiovascular regulatory centres as a semi-quantitative measure of activation. Depending on the outcome, we assumed we could pinpoint where central activation occurred, and by sequentially eliminating the players of the splenorenal reflex, ascribe the origin of central stimulation to either the kidney or the spleen. However, as with many scientific studies, our results proved to be more complicated than originally conceived. In retrospect, a number of modifications to this study would have clarified our results. Although demonstrating a central effect, we were unable to conclusively describe the pathways leading to central neural activation as a result of the splenorenal reflex.

The use of *c-fos* as a marker of neuronal activation is a well-established and valuable technique (11,18). Visual identification of the protein product Fos clearly marks neurons which have responded to a specific stimulus by an increased spike frequency – only neurons which have been strongly activated express the *c-fos* gene (11). However, as an independent method, it is subject to confounding factors leading to erroneous interpretation. Despite the presumed clarity of results based on the use of *c-fos*, extreme caution must be used during interpretation. Interpreting negative results can prove exceptionally difficult; over-stimulation of a neuron, for example, may activate intracellular mechanisms responsible for inhibiting *c-fos* expression (18). Used in conjunction with other techniques, such as retrograde or anterograde tracing (fluorescence), the target(s) of a neuron expressing Fos can be identified, clarifying the central pathways involved with the stimulus under investigation.

Immunohistochemical analysis of *c-fos* expression has inherent disadvantages. *c-fos* expression is rapidly induced by a broad range of stimuli (23), which is a potential hazard in interpreting results. It is difficult to control for every potential stimulus for neuronal activation, thereby making it difficult to conclude without a doubt a causal relationship to the intended stimulus. In the face of multiple stimuli, it would therefore be impossible to link a single stimulus to any activated neurons. Anesthesia is known to affect Fos expression (23). Sodium pentobarbital, which was used to prepare animals for fixation in the experiments of Chapter 3, is known to suppress Fos expression (23). In light of this, the duration of anesthesia before fixation was kept to a minimum (~15 min) but may still have had a lingering effect. Stress (due to handling or exposure to a novel environment) is known to stimulate Fos expression (23). Inherent to studies quantifying central *c-fos*, we were faced with the inability to use experimental (occluded) animals as their own non-occluded control, making statistical comparisons across subjects difficult. However, to minimize all of these extraneous factors, every effort was made to ensure that control groups were treated in the exact manner as experimental groups (aside from splenic vein occlusion), thereby creating a consistent "background" effect on *c-fos* expression.

4.3. POSSIBLE FUTURE STUDIES

The results presented in Chapter 3 point to an interesting role of the splenorenal reflex (and splenic baroreceptors in general) in central neural regulation of the cardiovascular system. Further studies are required to clarify the exact nature by which the spleen influences cardiovascular regulation. Double-labeling (ex. with tyrosine hydroxylase for noradrenaline (NA) content) could be employed to identify the composition of neurons activated by the splenorenal reflex. Retrograde and/or anterograde fluorescence tracing could be used to identify the target(s) of activated neurons. A sinoaortic-denervated and/or vagotomized group consisting of controls and splenic vein-occluded animals could be included to determine the baroreceptor-mediated effects of the splenorenal reflex. Inhibition of the effects of angiotensin II (ATII) with an angiotensin converting enzyme (ACE) inhibitor or ATII-receptor blocker would help elucidate the relative role of ATII in mediating the splenorenal reflex central effects.

We have yet to determine whether input from the spleen reaches the brain directly. It is tempting to speculate that the central effects observed with splenic vein occlusion result, at least in part, from a reflex inhibition of cardiac afferents to the brainstem. It would be interesting to examine the effect of splenic vein occlusion on cardiac receptors (ie. atrial volume receptors) to determine whether the central effects observed are secondary to a reduction in cardiac preload and subsequently reduced atrial receptor discharge. Venous congestion in the spleen (indicative of splanchnic congestion) produces central hypovolemia through pooling of a large volume of blood in the splanchnic bed. This would naturally lead to unloading of atrial volume receptors to trigger reflex salt and water retention to bring blood volume back up. Preliminary speculations point to a spleno-intestinal reflex by which increased splenic afferent nerve activity inhibits sympathetic outflow to the mesentery, thereby reducing splanchnic vasoconstriction and blood mobilization, leading to decreased venous return and cardiac preload. This hypothesis could be tested by occluding the splenic vein and observing the effect on atrial dimensions (through ultrasound imaging) or by directly measuring right atrial pressure or atrial receptor discharge following splenic vein occlusion in an acute rat preparation. The role of this proposed reflex appears to be strictly pathophysiological, serving to exacerbate splanchnic pooling in portal hypertension. The underlying physiological role remains to be determined.

4.4. CONCLUSIONS

In conclusion, the results comprising this thesis support the role of the spleen in regulating cardiovascular homeostasis, specifically through the input provided by splenic afferent nerves. The established roles of the spleen in immune function, in regulating intravascular blood volume, and in controlling systemic blood pressure are all indicative of the importance of the spleen for human survival. The clinical significance of this dissertation arises from furthering our understanding of the physiology and pathophysiology of the spleen. Evidence presented for the physiological significance of the spleen in cardiovascular regulation and its role in the pathogenesis of portal hypertension implies a risk associated with splenectomy to be considered in cases of elective surgery, and points to a future direction in the treatment of portal hypertension.

BIBLIOGRAPHY

1. Almeida, F. A., M. Suzuki, and T. Maack. Atrial natriuretic factor increases hematocrit and decreases plasma volume in nephrectomized rats. *Life Sci* 39: 1193-9, 1986.
2. Andrew, P. S. , Y. Deng, R. Sultanian, and S. Kaufman. Nitric oxide increases fluid extravasation from the splenic circulation of the rat. *Am J Physiol Regul Integr Comp Physiol* 280: R959-67, 2001.
3. Brookes, Z. L. and S. Kaufman. Effects of atrial natriuretic peptide on the extrasplenic microvasculature and lymphatics in the rat in vivo. *J Physiol* 565: 269-77, 2005.
4. Calaresu, F. R., J. C. Tobey, S. R. Heidemann, and L. C. Weaver. Splenic and renal sympathetic responses to stimulation of splenic receptors in cats. *Am J Physiol* 247: R856-65, 1984.
5. Chen, A. and S. Kaufman. Splenic blood flow and fluid efflux from the intravascular space in the rat. *J Physiol* 490 (Pt 2): 493-9, 1996.
6. Chevendra, V. and L. C. Weaver. Distribution of splenic, mesenteric and renal neurons in sympathetic ganglia in rats. *J Auton Nerv Syst* 33: 47-54, 1991.
7. de Bold, A. J., H. B. Borenstein, A. T. Veress, and H. Sonnenberg. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci* 28: 89-94, 1981.
8. Deng, Y., L. Copeman, and S. Kaufman. A novel natriuretic, hypotensive factor derived from the spleen. *Life Sci* 57: 1647-53, 1995.
9. Deng, Y. and S. Kaufman. Splenorenal reflex regulation of arterial pressure. *Hypertension* 38: 348-52, 2001.

10. DiBona, G. F. and L. L. Rios. Renal nerves in compensatory renal response to contralateral renal denervation. *Am J Physiol* 238: F26-30, 1980.

11. Dragunow, M. and R. Faull. The use of c-fos as a metabolic marker in neuronal pathway tracing. *J Neurosci Methods* 29: 261-5, 1989.

12. Elfvin, L. G., H. Aldskogius, and J. Johansson. Splenic primary sensory afferents in the guinea pig demonstrated with anterogradely transported wheat-germ agglutinin conjugated to horseradish peroxidase. *Cell Tissue Res* 269: 229-34, 1992.

13. Ermirio, R., P. Ruggeri, C. Molinari, and L. C. Weaver. Somatic and visceral inputs to neurons of the rostral ventrolateral medulla. *Am J Physiol* 265: R35-40, 1993.

14. Greenway, C. V. Role of splanchnic venous system in overall cardiovascular homeostasis. *Fed Proc* 42: 1678-84, 1983.

15. In Groom, A. C. and E. E. Schmidt, eds. Chapter 4: Microcirculatory blood flow through the spleen. London, Chapman and Hall Medical. 1990, 45-102.

16. Hainsworth, R. The physiological approach to cardiovascular reflexes. *Clin Sci (Lond)* 91 Suppl: 43-9, 1996.

17. Herman, N. L., D. R. Kostreva, and J. P. Kampine. Splenic afferents and some of their reflex responses. *Am J Physiol* 242: R247-54, 1982.

18. Hoffman, G. E., M. S. Smith, and J. G. Verbalis. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Front Neuroendocrinol* 14: 173-213, 1993.

19. Kaufman, S. Role of spleen in ANF-induced reduction in plasma volume. *Can J Physiol Pharmacol* 70: 1104-8, 1992.

20. Kaufman, S., P. Andrew, R. Sultanian, and Y. Deng. Adrenomedullin increases fluid

extravasation from the splenic circulation of the rat. *J Physiol* 534: 527-33, 2001.

21. Kaufman, S. and Y. Deng. Effect of splenic extract on plasma volume and renal function in the rat. *Life Sci* 65: 2653-62, 1999.

22. Koyama, S., F. Sawano, Y. Matsuda, Y. Saeki, T. Shibamoto, T. Hayashi Jr, Y. Matsubayashi, and M. Kawamoto. Spatial and temporal differing control of sympathetic activities during hemorrhage. *Am J Physiol* 262: R579-85, 1992.

23. Krukoff, T. L. Expression of c-fos in studies of central autonomic and sensory systems. *Mol Neurobiol* 7: 247-63, 1993.

24. Krukoff, T. L. and Y. Zheng. Effects of renal denervation and reinnervation on ganglionic gene expression of neurotransmitter proteins and c-fos in rat. *Brain Res Mol Brain Res* 19: 287-92, 1993.

25. Li, Y., D. Song, Y. Zhang, and S. S. Lee. Effect of neonatal capsaicin treatment on haemodynamics and renal function in cirrhotic rats. *Gut* 52: 293-9, 2003.

26. Lin, P. W. and Y. S. Shan. Effects of splenectomy and splenic artery ligation on the portal pressure in portal hypertensive rats. *J Surg Res* 53: 621-4, 1992.

27. Loftus, J. P., D. M. Nagorney, D. Ilstrup, and A. R. Kunselman. Sinistral portal hypertension. Splenectomy or expectant management. *Ann Surg* 217: 35-40, 1993.

28. Lorton, D., D. L. Bellinger, S. Y. Felten, and D. L. Felten. Substance P innervation of spleen in rats: nerve fibers associate with lymphocytes and macrophages in specific compartments of the spleen. *Brain Behav Immun* 5: 29-40, 1991.

29. Lundberg, J. M., A. Anggard, J. Pernow, and T. Hokfelt. Neuropeptide Y-, substance P- and VIP-immunoreactive nerves in cat spleen in relation to autonomic vascular and volume control. *Cell Tissue Res* 239: 9-18, 1985.

30. McClusky, D. A. 3rd, L. J. Skandalakis, G. L. Colborn, and J. E. Skandalakis. Tribute to a triad: history of splenic anatomy, physiology, and surgery--part 1. *World J Surg* 23: 311-25, 1999.

31. Meckler, R. L. and L. C. Weaver. Characteristics of ongoing and reflex discharge of single splenic and renal sympathetic postganglionic fibres in cats. *J Physiol* 396: 139-53, 1988.

32. Meckler, R. L. and L. C. Weaver. Persistent firing of splenic and renal afferent nerves after acute decentralization but failure to produce ganglionic reflexes. *Neurosci Lett* 88: 167-72, 1988.

33. Ohta, M., M. Hashizume, K. Tanoue, S. Kitano, K. Sugimachi, and K. Yasumori. Splenic hyperkinetic state and splenic artery aneurysm in portal hypertension. *Hepatogastroenterology* 39: 529-32, 1992.

34. Parks, D. A. and E. D. Jacobson. Physiology of the splanchnic circulation. *Arch Intern Med* 145: 1278-81, 1985.

35. Ralevic, V. Splanchnic circulatory physiology. *Hepatogastroenterology* 46 Suppl 2: 1409-13, 1999.

36. Song, D., H. Liu, K. A. Sharkey, and S. S. Lee. Hyperdynamic circulation in portal-hypertensive rats is dependent on central c-fos gene expression. *Hepatology* 35: 159-66, 2002.

37. Sultanian, R., Y. Deng, and S. Kaufman. Atrial natriuretic factor increases splenic microvascular pressure and fluid extravasation in the rat. *J Physiol* 533: 273-80, 2001.

38. Weaver, L. C., H. K. Fry, and R. L. Meckler. Differential renal and splenic nerve responses to vagal and spinal afferent inputs. *Am J Physiol* 246: R78-87, 1984.

APPENDIX

APPENDIX A.

GENERAL SURGICAL PROCEDURE FOR VESSEL CANNULATIONS

APPENDIX B.

CONSTRUCTION OF VASCULAR BALLOON OCCLUDER

APPENDIX C.

PROCEDURE FOR ACUTE IMPLANTATION AND USE OF TRANSONIC FLOW PROBES

APPENDIX D.

ACUTE SURGICAL PROCEDURE FOR EXTRACELLULAR SPLENIC AFFERENT NERVE RECORDING

APPENDIX E.

PROCEDURE FOR CHRONIC IMPLANTATION AND USE OF PA-C10 TELEMETRIC PRESSURE TRANSDUCER

APPENDIX F.

INTRACELLULAR PATHWAYS FOR C-FOS EXPRESSION

APPENDIX A

GENERAL SURGICAL PROCEDURE FOR VESSEL CANNULATIONS

Catheters used for cannulation were rinsed/flushed with distilled water prior to surgery (ie. prior to sterilization for chronic surgeries). Cannulae were prepared for insertion by beveling their ends with a sharp scalpel (polyethylene) or scissors (Silastic/MicroRenethane). For chronic cannulations, such as the abdominal aorta, catheters were packaged for and sterilized by gas sterilization (University of Alberta Hospital Central Sterile Services, Edmonton, AB, Canada). At the time of surgery, cannulae were connected to 1 ml syringes filled with heparinized saline (10,000 K.I.U/L).

Cannulation	Tubing	Inner diameter (mm)	Outer diameter (mm)
Femoral artery	PE-50	0.58	0.97
Femoral vein	Silastic	0.51	0.94
Gastric artery	PE-10	0.28	0.61
Gastric vein	Silastic	0.30	0.64
Abdominal aorta	MRE-033	0.36	0.84

In preparation for any acute or chronic surgical procedure, rats were anesthetized with sodium pentobarbital (62 mg/kg body wt, ip; MTC Pharmaceuticals, Cambridge, ON). In acute procedures, anesthesia was maintained with a long-acting barbiturate, Inactin (thiobutabarbital sodium, 80 mg/kg body wt, sc; Sigma, St. Louis, MO). For chronic procedures, supplementary doses of sodium pentobarbital were administered as necessary to maintain the surgical plane. Each animal received a dose of atropine sulphate (0.10 mg/kg body wt, im; Sabex, Boucherville, QC, Canada) to minimize bodily secretions under anesthesia. Anesthetic-induced hypothermia was prevented using Delta-phase heating pads

(Braintree Scientific, Braintree, MA). All surgical sites were shaved and cleaned with iodine surgical scrub solution.

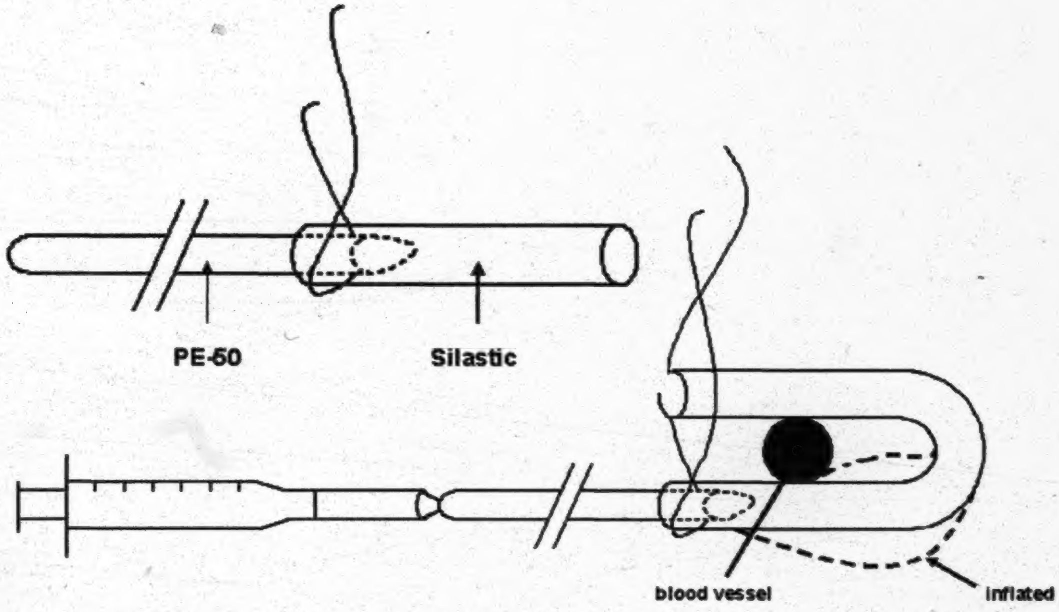
For occlusive cannulations, the vessels of interest (ie. femoral artery and vein, and gastric artery and vein) were identified and separated from their arterial/venous counterpart using blunt dissection. Once cleared, 4-0 silk sutures were inserted at each end of the freed areas of both the artery and vein. The distal ligature was tied tightly to occlude the vessel, while the proximal (midline) ligature was tied in a loose knot. Hemostats were clamped to each suture and used to gently retract and temporarily prevent blood flow in the vessel. For the larger femoral vessels, sharp curved forceps were used to lift the artery or vein into a tent shape; spring scissors were used to make a small incision in the vessel, through which the catheter could be inserted. For the smaller gastric vein and artery, a 27G or 30G needle (respectively) was bent and used to puncture and subsequently lift a hole in the vessel through which the catheter could be inserted. After drawing blood to ensure successful cannulation and to eliminate air from the line, hemostat retraction was released and the ligatures tied firmly around the catheters.

The procedure for chronic non-occlusive cannulation, such as the abdominal aorta, closely resembles that for occlusive cannulation of the gastric vessels. Retraction sutures were placed at two positions on the vessel above the bifurcation to the left and right femoral arteries, and below the renal artery. 4-0 silk sutures were placed in the adjacent psoas muscle to secure the cannula once inserted. After vessel retraction, the aorta was punctured with a bent 25G needle and MicroRenethane tubing inserted. Retraction was quickly released, and pressure placed on the point of insertion to prevent excessive blood loss while the vessel formed a seal around the cannula. Once a clot had formed, the cannula was tied in using the two silk 'stay' sutures and retraction sutures were removed.

APPENDIX B

CONSTRUCTION OF VASCULAR BALLOON OCCLUDER

A vascular balloon occluder was constructed using a 2 cm length of Silastic tubing (Dow Corning Corporation, Midland, MI; 0.51 mm ID, 0.94 mm OD) secured with 4/0 silk ligatures over the end of a 25 cm length of polyethylene tubing (PE-50; Becton Dickinson, Sparks, MD; 0.58 mm ID, 0.97 mm OD) connected to a 1 ml syringe filled with isotonic saline. While pinching the end of the Silastic tubing, the syringe was used to inflate the balloon several times to ensure integrity of the balloon. A silk ligature was used to tie off the balloon tubing. The free Silastic end of the balloon occluder was looped around the splenic vein or artery and tied. It was necessary to ensure the deflated balloon did not obstruct the vessel at baseline. The balloon occluder was secured to external supports to prevent accidental vessel occlusion for the duration of the acute *in vivo* experimental protocol.

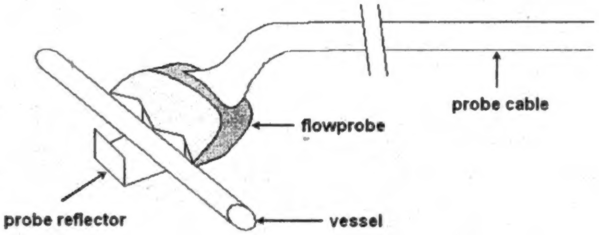


APPENDIX C

PROCEDURE FOR ACUTE IMPLANTATION AND USE OF TRANSONIC FLOW PROBES

The spleen and its vasculature were exposed via a midline laparotomy. The spleen was freed by cutting the surrounding peritoneum and ligaments immobilizing it to the stomach and diaphragm. The splenic vasculature was isolated by ligating all vessels to and/or from the spleen, except the splenic artery and vein. Small sections of the splenic artery and vein were gently and meticulously cleared of fatty and pancreatic tissue. The splenic vein and/or artery were slipped into the window of a 1RB and/or 0.5V series transit-time flow probe. The probes and vessels were adjusted such that the vessels lay unimpeded and centered within the acoustic window. In acute experiments involving simultaneous nerve recording, acoustic coupling was provided by mineral oil.

The Transonic Systems' Animal Research T206 dual channels flowmeter uses transit-time ultrasound to directly measure volume flow in a blood vessel. The flow probe consists of two ultrasonic transducers and a fixed acoustic reflector. The two transducers are located on one side of the vessel of interest, and the reflector is midway between them on the opposite side, therefore passing ultrasonic signals back and forth. In this way, upstream and downstream signals are transmitted through the vessel between the transducers and integrated twice on the reflective path to give an accurate measure of volume flow.



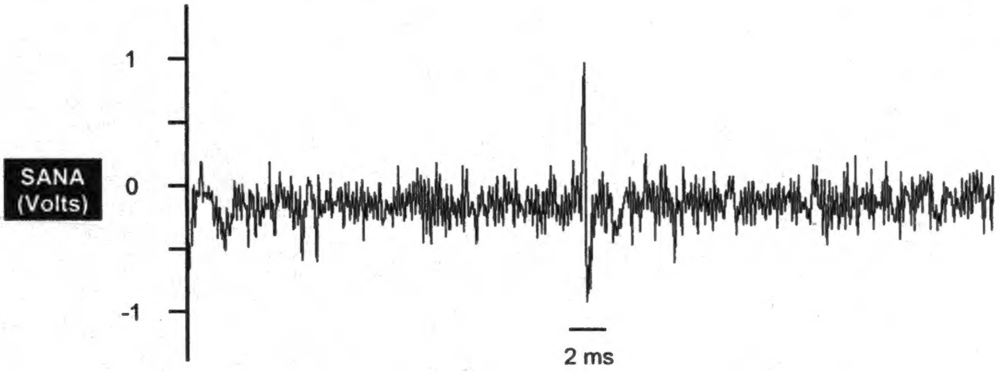
APPENDIX D

ACUTE SURGICAL PROCEDURE FOR EXTRACELLULAR SPLENIC AFFERENT NERVE RECORDING

A ventral midline abdominal skin incision was made with a No. 15 scalpel blade from xiphisternum to the lower abdomen. The linea alba was lifted with tissue forceps at the posterior end of the incision and a puncture wound into the abdominal cavity made with the scalpel. The abdominal incision was extended from the lower abdomen to xiphisternum along the linea alba, exposing the abdominal organs. Using two wet Q-tips, the stomach was retracted out of the abdominal cavity and reflected up on the rat's thorax and wrapped in saline-soaked gauze. Cutting the connective tissue between the stomach, spleen and liver allowed the liver to be gently tucked behind the gastric vessels for the duration of the experiment. Using a curved needle and silk sutures, the edges of the abdominal muscle layer were secured to a wire support ring, which created a cavity to house warm mineral oil required to electrically isolate the nerve.

The acute preparation area was kept as clean as possible to minimize aqueous fluid build up which could short out the nerve recording electrode. To do this, a 2x2 gauze was placed over the abdominal organs, except for the spleen and its accompanying vasculature (and pancreas), which were then laid on top. The spleen was covered with a small piece of gauze to prevent desiccation. The abdominal cavity was then filled with warm mineral oil. Using oil-soaked Q-tips, the pancreas was gently pulled away from the splenic vessels and the large lymph node at the base of the gastric vessels was pushed aside.

With extreme care to avoid damaging the splenic nerve, the splenic artery and vein were carefully separated to expose a section of the splenic nerve. Fine forceps were used to isolate and divide the splenic nerve for recording of afferent nerve activity. The nerve is generally located as a bundle between the artery and vein, although may appear as a network of nerve fibers surrounding the splenic artery. A section ~10-15 mm long was cleared with No. 5 fine forceps. The nerve was cut at the most proximal point and carefully dissected away from the artery and surrounding tissues using blunt dissection. Once freed, the nerve was left unaltered for 15 min, and then the cut end was carefully lifted onto the bipolar platinum recording electrode. Nerve activity was amplified and filtered (low frequency cutoff = 100 Hz; high frequency cutoff = 10,000 Hz) at a sample rate of 10,000 samples/s.

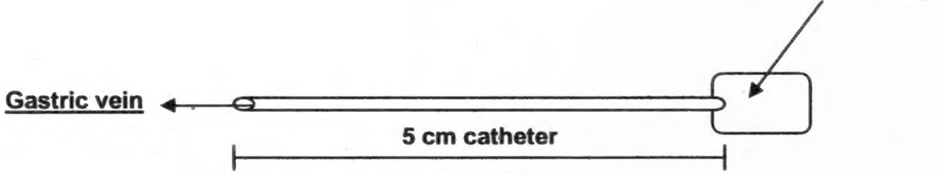


Representative compound action potential counted during analysis of *in vivo* extracellular nerve recording from a splenic afferent nerve fiber bundle (SANA, splenic afferent nerve activity).

APPENDIX E

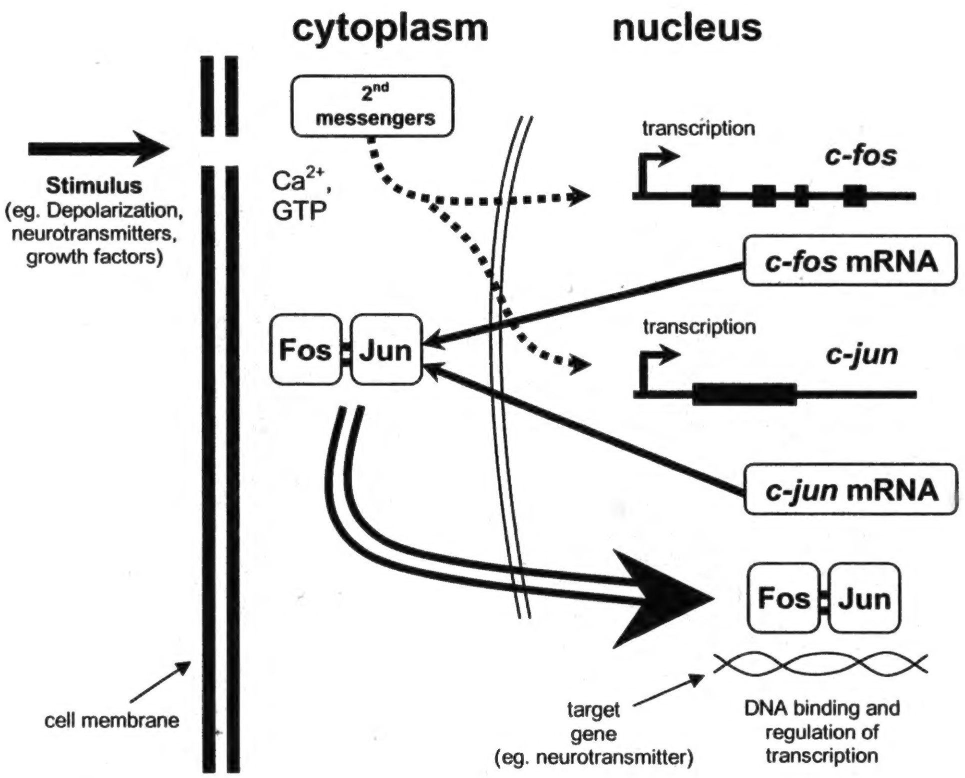
SURGICAL PROCEDURE FOR CHRONIC IMPLANTATION AND USE OF PA-C10 TELEMETRIC PRESSURE TRANSDUCER

The procedure for chronically implanting the PhysioTel PA-C10 telemetric pressure transducer (Data Sciences International, Arden Hills, MN) into the gastric vein of the rat closely resembles the procedure for occlusive gastric vein cannulation. Using aseptic technique under pentobarbital-induced anesthesia, a midline incision (~5 cm in length) was made to expose the abdominal cavity. Organs were gently moved aside, and the stomach freed to rest upside down on the rat's thorax. The gastric vein was isolated for cannulation by dissecting the connective tissue between the spleen, liver and stomach. The freed lobe of the liver was gently tucked behind the gastric vessels to clear the prep area. Two small areas of the gastric vein were carefully cleared using blunt, curved forceps, and two silk retraction ligatures threaded around the vessel. The uppermost ligature was tied off, while the bottom ligature was left untied during the cannulating process. After applying slight retraction to the vessel, a bent 30G needle was used to puncture the gastric vein, through which a catheter could be inserted. A PA-C10 transmitter catheter was inserted into the gastric vein and its tip advanced to the junction with the splenic vein for measurement of splenic venous pressure. Using two silk sutures, the body of the transmitter (1.1 cc, 1.4 g) was sewn to the abdominal wall ventral to the stomach and gastric vessels.



APPENDIX F

INTRACELLULAR PATHWAYS FOR C-FOS EXPRESSION


cytoplasm
nucleus
2nd messengers
Stimulus
(eg. Depolarization, neurotransmitters, growth factors)
Ca2+, GTP
transcription
c-fos
c-fos mRNA
Fos
Jun
transcription
c-jun
c-jun mRNA
Fos
Jun
cell membrane
target gene (eg. neurotransmitter)
DNA binding and regulation of transcription

Schematic illustrating the process by which transcription of immediate-early genes *c-fos* and *c-jun* leads to translation of the Fos-Jun protein complex within neuronal cells. An initial external stimulus causes neuronal depolarization and activation of intracellular messengers leading to transcription of the immediate-early genes, *c-fos* and *c-jun*. Messenger RNA (mRNA) is translated into the protein products, Fos and Jun, which combine to form a heterodimeric transcription factor capable of regulating downstream expression of other target genes. [Figure reproduced with permission from Dampney, R. A. and Horiuchi, J. Functional organisation of central cardiovascular pathways: studies using c-fos gene expression. Prog Neurobiol. 2003 Dec; 71(5): 359-84.]